Tulasi Satyanarayana
Sunil Kumar Deshmukh *Editors*

Fungi and Fungal Products in Human Welfare and Biotechnology

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Preface

The fungal kingdom is estimated at 2–4 million species, among which less than 5% have been formally described because of under sampling in fungal diversity studies [Ann. Rev. Microbiol. (2020) 74: 291–313]. The fungal kingdom has been organized based on genome sequencing into divisions, such as Ascomycota, Basidiomycota, Zygomycota, Glomeromycota, Blastocladiomycota, and Chytridiomycota. Fungi are capable of surviving in a great variety of growth conditions and biological interactions, and thus have significant economic impacts for humankind in many ways considering their effect on food losses through pre- and post-harvesting plant colonization, and human and animal diseases. Fungi are fundamental for biotechnological advancements, producing a vast majority of industrial enzymes useful in several different processes, and also in the production of organic acids, antibiotics, foods, and beverages [Fung. Biol. Biotechnol. (2020) 7: 5].

Fungal biology has emerged in the last decade as a very strong scientific field with a phenomenal surge in publications and understanding basic and applied biological processes. Traditionally, several fungal model systems have provided important biological discoveries commencing from George Beadle's hypothesis "one gene—one enzyme" using *Neurospora crassa* as a model system in 1941. Genetic transformation systems and genome sequencing are being expanded to a plethora of filamentous fungi making it possible to understand specific ecological interactions with insects, mammals, and plants revealing highly sophisticated mechanisms of commensalism and pathogenesis, such as those found in arbuscular mycorrhiza and fungal pathogens. The recent developments in CRISPR Cas9 technologies for filamentous fungi will provide amazing opportunities for understanding various processes in fungi [Appl. Microbiol. Biotechnol. (2019) 103: 6919–6932].

The product range of filamentous fungi is not just limited to citric acid and enzymes. Organic acids beyond citric acid are itaconate, galactarate, and several others. Itaconate could replace petroleum-based polyacrylic acid, which is a precursor for the polymer industry (absorbent polymers, polyester resins, synthetic latex), while galactarate could replace the current petroleum-based polyethylene terephthalate (PET) used in producing plastics.

In fact, the natural metabolic capabilities of filamentous fungi are extraordinarily diverse and unmatched in nature. Many companies around the globe are leveraging

the power of filamentous fungi, with major players in Europe that include: AB Enzymes, BASF, Bayer, Chr. Hansen, Dyadic International, DSM, DuPont, Kerry Group, Marlow Foods, Novozymes, Puratos, Syngenta, and Roal Oy. In a more recent endeavour, biologists, chemists, bioinformaticians, bioengineers, process engineers, and material scientists have collaborated to turn by-products and waste from agriculture and forestry into composite materials with the help of mushroom-forming fungi.

The use of fungi as a source of food has long history. These have been predominantly the mushrooms from supermarket shelves and foraging expeditions. Morels had been first successfully cultivated commercially in Sichuan in 2012; it expanded to more than 20 provinces in China. The highest yield has currently reached 15,000 kg/ha. Morel cultivation is characterized by its environmental friendliness, short cycle length, and high profit [Appl. Microbiol. Biotechnol. (2022) 106: 4401–4412]. However, the yield obtained is unstable which makes morel cultivation a high-risk industry.

There has recently been a move to create meat-like products from fungal mycelium grown in fermenters, rather than the solid fruiting bodies. This has allowed the introduction of ascomycetes, traditionally used as flavour modifiers in such foods as blue cheese, to enter the food chain as convincing meat substitutes. The earliest established of these companies is Marlow Foods, using *Fusarium venenatum* under the trade name Quorn™, but several other companies have recently shown an interest in this area, including Mycorena (using *A. oryzae*), Sustainable Bioproducts (using *F. oxysporum*) and MycoTechnology, using the basidiomycete *Lentinula edodes*.

During the colonization of substrates, hyphae bind the organic particles together, while degrading them simultaneously. A composite material is obtained, consisting of a bulk of organic substrate bound together by the hyphal network, by inactivating the fungus before the substrate is degraded (e.g. by drying or by heat inactivation). Pure fungal materials are obtained by complete degradation of the substrate or removing the fungal skin from the substrate. Both pure and composite mycelium can be used for different applications. Stopping fungal growth during colonization of the substrate results in materials with properties similar to that of expanded polystyrene or other foams. Such products can be used as packaging material or for heat or acoustic insulation.

Mushroom-based materials have the potential for uses in place of leather, textiles, and some plastics. The strength of pure mycelium of *Schizophyllum commune* is similar to that of natural materials, such as wood and leather. The flexibility and aesthetics of mushroom materials were first appreciated by artists and designers who used them to grow living art works. Similar to cement and plaster, mycelia will bind, harden, and set into a variety of solidified configuration.

In order to shift away from a fossil-based economy and mitigate climate change triggered by the continuous industrial overproduction of CO_2, many countries around the world, including those of the European Union, support the transformation to a renewable, bio-based, and resource-efficient economy.

Stronger mutual collaborations between scientists, engineers, artists, designers and industrial stakeholders, and vivid communication with the general public and policymakers will only ensure that the inter- and transdisciplinary science on fungi will create a path towards innovative breakthroughs that will create a sustainable economic future based on fungal cell factories for years to come.

The book entitled *Fungi and Fungal Products in Human Welfare and Biotechnology* was planned and executed with the main objective of reviewing developments on different aspects of fungi and fungal products useful in human welfare and biotechnology. The book is divided into four parts: Part I entitled "Plant Growth and Sustainability" includes five chapters, Part II entitled "Fungal Metabolic Products" consists of five chapters (6–10), Part III titled "Fungi in Food Biotechnology" contains three chapters (11–13), and Part IV entitled "Fungal Enzymes and Other Biotech Products" consists of eight chapters.

The chapters of the book have been contributed by the expert mycologists and biotechnologists from India and abroad. The contributors have readily accepted our invitation and submitted well-written chapters within the prescribed timelines. We wish to thank all authors for their hard work in reviewing the developments and presenting in a smooth and readable format.

We wish to acknowledge the efforts made by Springer Nature in publishing the book very efficiently in a short span of time.

New Delhi, India — Tulasi Satyanarayana
Pune, Maharashtra, India — Sunil Kumar Deshmukh

Contents

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About the Editors

Tulasi Satyanarayana has been UGC-Faculty Fellow and Professor Emeritus at the Division of Biological Sciences and Engineering, Netaji Subhas University of Technology, New Delhi, after superannuating from the Department of Microbiology, University of Delhi South Campus, New Delhi, as Professor and Head, in June 2016. He has over 275 scientific papers and reviews, 14 edited books, and 3 patents to his credit. He is a fellow of the National Academy of Agricultural Sciences (NAAS), Academy of Microbiological Sciences (AMSc), Biotech Research Society (I) [BRSI], Mycological Society of India (MSI), and Telangana Academy of Sciences. He is a recipient of Dr. Manjrekar Award of AMI, Dr. Agnihotrudu Memorial Award of MSI, and Malaviya Memorial Award of BRSI for his significant contributions. He has over 45 years of research and teaching experience and has mentored 30 scholars for Ph.D. He was the president of AMI and MSI. He successfully executed 17 major research projects sanctioned by various Govt. agencies. He had collaborative Indo-German and Indo-Japanese research projects. His research efforts have been focused on understanding the diversity and applications of yeasts, thermophilic fungi and bacteria and their enzymes, metagenomics, carbon sequestration employing extremophilic bacterial carbonic anhydrases and bioethanol production from lignocellulosics employing microbial enzyme cocktails.

Sunil Kumar Deshmukh is Scientific Advisor to Greenvention Biotech, Uruli-Kanchan, Pune, India, and Agpharm Bioinnovations LLP, Patiala, Punjab, India. He is a veteran industrial mycologist; he spent a substantial part of his career in drug discovery at Hoechst Marion Roussel Limited [now Sanofi India Ltd.], Mumbai, and Piramal Enterprises Limited, Mumbai. He has also served TERI-Deakin Nano-Biotechnology Centre, TERI, New Delhi, and as an Adjunct Associate Professor at Deakin University, Australia. He has to his credit 8 patents, 145 publications, and 17 books on various aspects of fungi and natural products of microbial origin. He is a president of the Association of Fungal Biologists and a past president of the Mycological Society of India (MSI). He is a fellow of Mycological Society of India, the Association of Biotechnology and Pharmacy, the Society for Applied Biotechnology, and Maharashtra Academy of Science. Dr. Deshmukh is associate

editor of Frontiers in Microbiology series editor of *Progress in Mycological Research* published by CRC Press. Dr. Deshmukh serves as a referee for more than 20 national and international journals. He has approximately four decades of research experience in getting bioactives from fungi and keratinophilic fungi.

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Part I

Plant Growth Promotion and Sustainability

Arbuscular Mycorrhizal Fungi: Role as Biofertilizers, Technology Development, and Economics

1

Karuna Sharma, Sarda Devi Thokchom, Samta Gupta, and Rupam Kapoor

Abstract

In pursuance of feeding the burgeoning population and achieving global food and nutrition security in the context of climate change, there is a pressing need to usher toward climate-smart, sustainable agriculture that is more resistant and resilient toward environmental abnormalities. In this regard, there is a progressively increasing recognition of the integral importance of rhizosphere in ecological sustainability, including the most widespread plant-symbiotic association, i.e., mycorrhiza. The endeavor for sustainability of agriculture is to provide enhanced crop production without defacing other allied, yet important, aspects of global ecosystems. This chapter dwells on how AMF (arbuscular mycorrhizal fungi) technology, which exploits AMF-plant interactions, is a potential way forward for achieving this goal. After placing emphasis on the ecosystem services generated by AMF and their direct role in plant nutrition, stress tolerance, and soil microbial diversity, the chapter provides an economic appraisal of the mycorrhizal technology and highlights the existing constraints and lacunae in the technology and its dissemination. The need for economic valuation of mycorrhizal technology and constraints associated therewith are also stressed along the chapter. The existing state of affairs of AMF technology and possible research trajectories that can be taken up are also discussed herein. The chapter also calls attention to the need for more applied research on the possible combination and assembly of AMF with other beneficial microbes and host genotype. Finally, future research goals aimed at maximizing the benefits of AMF technology are also discussed.

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Keywords

Arbuscular mycorrhizal fungi · Mycorrhizal technology · Sustainable agriculture · Biofertilizers · Economic assessment

1.1 Introduction

Fungi are among the most primitive and largest life-forms that serve as the principal agents of organic matter decomposition, thereby contributing to carbon cycle sustenance. As mycorrhizal association of roots, they provide the primary mechanism for the capture of nutrients used by plants, hence contributing to the green cover on earth. The most important and widespread mutualism that exists on earth is between soil fungi, belonging to phylum Glomeromycota, and ca 80% of extant land plants, which is in a symbiotic relationship called "mycorrhiza." The evolution of obligate root inhabiting fungi is one of the most important events in the process of terrestrialization of plants. DNA analysis and fossil preserves provide a tantalizing sight into the evolutionary history of this mutualistic plant-fungus relationship of great antiquity that dates back to 400–460 million years [1]. Based on their relationship with roots, mycorrhiza can be categorized as ectomycorrhizal or endomycorrhizal depending on whether or not they invaginate into the plant roots.

Since antiquity, this symbiotic group of fungi has been involved in multitudinous direct (accelerated nutrient acquisition, carbon reallocation, heavy metal immobilization, systemic tolerance toward pathogens) and indirect (photosynthesis stimulation, abiotic stress tolerance, refined soil health and conditioning) advantages to plants, with potential consequences to agricultural product quality, multi-trophic interaction networks, and soil quality [2]. In addition to the calibrated molecular dialogue with their hosts, the mycorrhizal fungi also interact with soil microflora, thereby rendering crucial ecosystem functions, and these interactions are further modulated by environmental (soil, climatic, ecosystem management) contexts. On all these accounts, the role of "vesicle- and arbuscule"-forming endomycorrhiza, i.e., vesicular arbuscular mycorrhiza (VAM), or nowadays known as AMF (arbuscular mycorrhizal fungi), has been widely acknowledged in considerably improving crop production and its performance while substantially shrinking the environmental footprint of production on large scale [3].

As a growing body of reports call to attention, the mushrooming global population that corresponds to an exponential increase in the food demands has become a grave area of concern under alarming conditions of climate change. This calls out for an increased need of biofortification and intensification of agriculture. Though agricultural intensification seems to be a good approach to subdue the impending food crisis, it also holds serious threat to the environmental health. This all the more necessitates the paradigm shift of agriculture toward a sustainable production system that espouses for environmentally favorable practices [4]. With the view of sustainable agriculture, employment of biofertilizers, especially AMF, is advocated as a promising approach by volumes of publications on the subject [4]. The relevance of

AMF in sustainable food production is far and wide. In regions where phosphorus depletion in soil is a serious problem, AMF-based products have an edge in terms of cost-effectiveness over the application of conventional fertilizers. They are not only more economical and eco-friendly but are also efficient enough to thwart the impact of low phosphorus availability in soil [5]. The relevance of AMF as natural biofertilizers is acclaimed by the fact that they enhance the nutrient and water acquisition of plants from the soil in lieu of photosynthates, hence have a relevant role in ecosystem functioning.

In order to make use of AMF technology in sustainable agriculture, effective combinations of crop plants and interacting fungal partners could conceivably deliver high plant performance and nutritional quality under all combinations of soil and climate. However, much remains ambiguous regarding the specified mechanisms that influence these processes. On that note, this chapter highlights the benefits proportionated by AMF in intensification of sustainable agriculture, its role as biofertilizers, the status quo and development of AMF technology, and its cost economics. The review emphasizes on the possibilities and prospects of transfer of AMF technology into commercial practices while dwelling on the problems linked to quality inoculum production. Along this chapter, a holistic view on the benefits of AM symbiosis along with components, applications, and bottlenecks of mycorrhizal technology is presented.

1.2 Role of AMF as Biofertilizer in Sustainable Agriculture

The term "biofertilizer" [6] can be interpreted in numerous ways. By definition, biofertilizers are microbial isolates, used individually or in amalgamation, that have the potential to endorse growth and development of plants [7]. They have an edge over chemical fertilizers as microbes can form symbiotic association with plant roots and directly help in plant nutrition by readily converting complex organic compounds into simple ones, making them easily accessible to plants. In addition, chemical fertilizers are expensive to both farmers and the environment. With this, biofertilizers have gained attention with each passing day. Since AMF epitomize a key connection between soil mineral nutrients and plants, they are duly acknowledged for their role as biofertilizers. The biofertilizer properties of AMF are frequently ascribed to their ability to (i) positively influence the ecosystem, (ii) interact synergistically with other beneficial microorganisms, (iii) improve tolerance to a/biotic stresses, and (iv) upsurge bioavailability of mineral nutrients, especially phosphorus and nitrogen [8, 9].

1.2.1 Ecosystem Services of Arbuscular Mycorrhizal Fungi

Restoration of ecosystem is one of the fundamental themes of global environmental policies [10, 11]. One of the targets laid by UN Convention on Biological Diversity for the years "2011–2020" is to reinstate at the minimum 15% of the degraded

Table 1.1 Overview of ecosystem services provided by AMF

S. no.	AMF function	Ecosystem services	Reference
1.	Modification of root architecture and development of ramified common mycorrhizal network popularly called the wood wide web	Increased plant anchorage to soil Promotion of soil aggregation and stability Transfer of carbon, nutrients, water, and defense compounds	[30]
2.	Increasing nutrient and water acquisition by plants especially from the regions otherwise not accessible to plants	Increase plant growth and development while minimizing the need of external fertilizer amendments	[31]
3.	Secretion of the wonder protein, glomalin, into the soil	Improve the texture and structural stability of soil as well as water retention property of soil	[32–34]
4.	Protection against notorious pathogens	Intensified tolerance/resistance of plants against pathogens while reducing physicochemical inputs	[35, 36]
5.	Buffering against abiotic stress	Improved tolerance of plants against salinity, drought, heavy metal pollution, mineral nutrient depletion, and temperature stress	[37–41]
6.	Modulation in plant physiological and metabolic parameters	Regulation of plant growth and development and improved quality of plant products for human health and welfare	[42–45]

ecosystems worldwide [12]. AMF, being keystone microbes with multitudes of ecosystem roles, are valued for ecological restoration (Table 1.1). Their ability to improve plants' nutrient and water relation increases the survivalism and establishment of plants in fields [13, 14]. They play a vital role in stabilizing soil structure as well [15]. During AM development, the hyphae of fungal symbionts grow out of host root system into the rhizosphere and form an intricately ramified network [16, 17]. This branched network represents approximately 50% of the total fungal hyphae in soil, thus contributing majorly to the soil microbial biomass [18, 19]. The hyphal network reportedly has the capacity to bind soil particles and refine its structure. Additionally, they secrete "sticky" hydrophobic proteins, known as glomalin, that confer stability of soil and can retain water [18, 20]. The consolidated effect of the wide-ranging hyphal network and glomalin is regarded to play a vital role in stabilizing soil aggregates, thereby resulting in increased soil quality and structural stability [20]. They further promote aggregation of soil by manipulating microbial population that can promote formation of soil masses [21, 22]. Thus, AMF improve both aggregation and stability of soil. Soka and Ritchie [23] reported that AMF can stabilize soil up to 5 months following the death of their host plants.

Agricultural practices such as fertilization, monoculture, or excessive plowing have often been reported to adversely influence the diversity and abundance of AMF in soil [24]. A decrease in the fungal population can negatively affect stability of soil and subsequently intensify the menace of soil erosion, whose consequence must not

be taken lightly. For instance, in the UK, erosion of agricultural soil resulted in an estimated productivity loss scaling up to 9.99 million Euros per year [25]. On a human timescale, as soil is a nonrenewable resource, the repercussions of erosion are every so often cumulative and irreversible in most instances.

The status of mycorrhizal fungi and soil fertility also affect the plant species establishment [26]. It is theorized that mycorrhizal fungi are drivers and passengers, as well, of plant community succession [27]. In the course of ecological succession, while the status of AMF diversity governs the structure of the plant community of a specific site, the plant community also exerts its influence on AMF diversity and by doing so, further determines the composition of upcoming seral plant community [26, 27]. Hence, if compatible associations between specific plant taxa and AMF are obligatory for the existence of the mutual symbionts, disappearance of such AMF species can confine distribution of plant species [27]. Plant-AMF feedback is an imperative concept that can explain AMF's role in succession. While positive feedback promotes the growth of primary successional communities, undesirable/negative feedbacks endorse replacement of plant species to drive succession [28]. Keeping in mind the perception that pioneer species often face harsh environmental condition during initiation of primary succession and mycorrhizal symbiosis delivers vital help for supporting growth of plants in extreme environments [29], AMF's role in primary succession is justified. Nevertheless, pioneer herbaceous species were reported to have very low rates of mycorrhizal colonization, and late-successional species evidently benefited from the symbiosis. These variations in the associations of AMF with pioneer and the late-successional species advocate that AMF may take part in an important and complicated, yet unfamiliar, ecological mechanism of primary succession [28].

1.2.2 Regulation of Soil Microbial Diversity and Interaction with Other Beneficial Microbes

The hyphae of AMF and plant roots are the most copious source of carbon in soil [46]. Hence, they increase the energy supply for soil microbes to flourish. Rillig [21] documented that by influencing establishment of plant communities, AMF also regulate microbial communities of soil. Moreover, exudates of fungal hyphae can also stimulate growth of microorganisms found in the mycorrhizosphere. Nevertheless, the consequence is variable. While some microbes may get stimulated by the exudate, others can get inhibited [47]. Thus, mycorrhizal fungi may surge the diversity and population of microbes, which are favorable for plants' growth.

Mycorrhizal fungi also interact synergistically with other beneficial fungi such as phosphate-solubilizing fungi and decomposer fungi [48]. Therefore, presence of AMF is regarded to change subterranean litter decomposition rate owing to change in the biochemical profile in rhizosphere and interplay with decomposer fungi [48]. Phosphate-solubilizing fungi reportedly had moderate impact on plant nutrition on the condition that they were introduced alone and greatest effect was observed when they were introduced together with AMF corroborating the synergistic

interaction between the two microbes [49]. Moreover, mycorrhizal fungi are also reported to act antagonistically with root and leaf pathogens [48, 50].

Mycorrhizal fungi are also reported to interact synergistically with each other. Several studies reported a greater effect of AMF when a consortium of inocula is introduced in comparison with single AMF inoculum [51]. Barea et al. [52], after continuous observation over the years, documented that employing consortia of native AMF spp. had the best effect on plant growth. A systemic review carried out on 306 studies by Hoeksema et al. [53] also showed that plant's response was considerably less when treated with only one AMF species vis-à-vis multiple AMF spp. inoculation. This may perhaps be credited to the synergistic interaction among several AMF spp. As AMF spp. vary in their hyphal growth patterns and the branching frequencies, the variances perhaps mirror diverse strategies and occupation of different niches within the soil [50].

1.2.3 Arbuscular Mycorrhizal Fungi and Plant Nutrition

The most appreciated contribution of AMF to plant performance is their capability to improve plants' nutrient uptake [54]. AMF deliver water and mineral nutrients to host plants in exchange of photosynthates [31]. Mycorrhizal hypha emerges from plant roots and acquires nutrients, which are otherwise not accessible to roots, from soil volumes [55]. Moreover, owing to their relatively thinner hyphae in comparison with the roots, the hypha pervades into the microsites of rhizospheric soil and by doing so expands the surface area of root to 100- or even 1000-fold [52]. The mycorrhizal hyphae colonize the root cortex and develop profusely branched structures called the arbuscules, the active site for nutrient exchange between the symbionts [50]. By harnessing AMF's potential to supply nutrients, the energy linked to fertilizer production as well as their application can be reduced considerably.

One of the major issues in optimizing efficient fertilization is reducing the amount of nutrients lost, through leaching, to the environment. AMF reduce leaching of nutrients not only by increasing the nutrient interception via formation of mycorrhizosphere but also by augmenting nutrient uptake, improving soil structure, and nurturing other microbial community [56]. AMF spp. differ in their capability to cut down on nutrient leaching, thus highlighting the importance of AMF diversity [57]. In connection with this, van der Heijden [58] demonstrated that increased nutrient leaching in fertilized agroecosystems is a result of higher nutrient content and reduced AMF diversity. Hence, AMF can benefit plants grown under nutrient-poor conditions [59]. In order to use AMF as biofertilizer for agricultural purposes, they must be incorporated in formulations, which will extend their shelf life, i.e., the time frame between its production and application in field (discussed in the subsequent section).

1.2.4 Plant Stress Tolerance Mediated by Arbuscular Mycorrhizal Fungi

Abiotic stresses hinder growth and development of plants, thereby resulting into extensive losses to agricultural productivity. Climate change and agrarian malpractices such as unwarranted utilization of pesticides and fertilizers have intensified the impacts of abiotic stress on plants and deteriorate the ecosystem as well. Drought, mineral diminution, heavy metals, salinity, and extreme temperature are some of the serious problems faced in various regions of the world [60]. It is foreseen that by 2025, one third of the arable land might disappear in Asia, two thirds in Africa, and one fifth in South America [61]. At this front, it is widely held that introduction of AMF in agriculture delivers tolerance to hosts against various stresses such as salinity, drought, heavy metals, temperature, herbivory, and diseases [40, 41]. Mycorrhizal fungi support growth and sustenance of host plants under various stresses by arbitrating complex communications between the symbionts and regulating a number of physio-biochemical processes resulting in increased photosynthetic rate, osmotic adjustment, stomatal regulation, and enhanced antioxidant system that help thwart the negative impacts of the stresses on plants [13, 62].

Mycorrhiza also support establishment of plants in heavy metal-contaminated soil by strengthening the defense system of host plants. Various reports in literature revealed the AMF-mediated impacts on accumulation of heavy metals in host plants [40, 63]. They immobilize heavy metal in the fungal hyphae, fix them in cell wall, sequester in vacuoles, or may chelate in the cytoplasm with other substances, thereby reducing the toxicity of metals in plants [64]. Furthermore, enhanced uptake of important immovable nutrients such as P, Zn, and Cu results in increased plant biomass, thereby leading to dilution of the metals in plant tissues [65, 66]. In summary, several processes that bring about metal tolerance by AMF include immobilization of metal compounds, adsorption to fungal chitin, chelation inside the fungal structures, and dilution effect caused by increased plant biomass.

1.3 Arbuscular Mycorrhiza: Technology Components, Development, and Constraints

Mycorrhizal technology can be defined as the set of measures to optimize local wealth and diversity of AMF in relation to functioning for building sustainable ecosystems. “Optimization” in this context is implied as increasing AM-induced benefits (in terms of yield and sustainability) at the farm level so as to obtain its full theoretical potential [67].

The main focus of applied research on AMF in sustainable agriculture could be centered on the development of mycorrhizal technology. Apparently, different techniques of production and application of AMF inocula are recognized as the essence of mycorrhizal technology [68], clearly addressing the decrease in AMF abundance and diversity in the agricultural fields. The conceptualization of framework of mycorrhizal technology should be premised on a concrete understanding of

the ways of achieving resource-efficient agriculture with smaller footprints and minimal environmental impacts [67].

1.3.1 Components of Mycorrhizal Technology

As proposed by Rillig et al. [67], there are five components of mycorrhizal technology, viz., (i) surveillance, (ii) management, (iii) database tools, (iv) plant breeding, and (v) mycoengineering. Surveillance refers to close monitoring and assessment of diversity of AMF in the field and its abundance in roots and soil. Management refers to (i) agronomic practices (tillage, application of fertilizers, irrigation patterns, and crop rotation) that can potentially influence the wealth and abundance of AMF species in the field and (ii) targeted approaches such as inoculation of plants with mycorrhizal inoculum or with that of mycorrhiza helper bacteria (MHB). Database tools represent an archive or repository of information regarding mycorrhizal abundance and functioning linked with site-specific parameters (geographical, topographical, edaphic, etc.). Plant breeding is a critical component of mycorrhizal technology as AM associations are of obligate nature and mycorrhizal fungi cannot be multiplied without a suitable host. Engineering of mycorrhizal communities, popularly known as "mycoengineering," represents an approach predicated in community ecology with an enterprise of developing and propagating the members of AMF with desirable attributes [67]. Inroads toward the establishment and progression of the components of mycorrhizal technology have been developed; however, demands for research and development are still varying.

The development of mycorrhizal technology is thus a multicomponent interactive approach. Agricultural management practices, for instance, stringently monitoring the nutrient stoichiometry in agrosystems, including the quantity and ratios, could be very effective in modulating the mycorrhizal sprawl in the agricultural fields, as AMF phylotypes are very susceptive of nutrient quotient in the soil [69]. Albeit real-time monitoring of diversity, abundance, and functioning of AMF is currently a huge bottleneck of this technology, but if successful, it would permit the detection of inimical agricultural practices, allowing explicit response at field level. When recorded across multiple fields and different time spans, these data can be deposited in the database, so that iterative optimization of local management intervention can be ensured. Selection of crop cover genotype is a parameter that directly influences the quality of host type for symbiosis, and thus, AMF abundance. In this context, collaboration of mycorrhizologists with plant breeders provides an enormous opportunity to select most fit genotypes that effectively get colonized with different fungal strains [67].

Microbial community engineering, also known as "mycoengineering," is a rapidly progressing field in microbial ecology. The technique is grounded on the rapid and robust screening followed by selection of the outperforming candidates over multiple microbial generations. Engineering the entire consortia of microbes associated with AMF is very promising in fostering the next green revolution, as it is known that interaction with other soil microorganisms is very contributive in AM

functionality and vice versa [67]. Some of these microbes are synergistic with mycorrhizae in improving plant growth and health [70] and may also trigger root colonization by AMF [71]. Several AMF species are also reported to synthesize certain organic metabolites, volatile and nonvolatile compounds, to lure specific bacteria [72]. For instance, benzoxazinoid metabolites were found increasingly produced in root exudates of wheat under the influence of AMF, thereby eliciting chemotaxis in plant growth-promoting rhizobacteria (PGPR) [72]. This points out that interaction of AMF with a plethora of soil-dwelling microflora renders them capable of augmented delivery of nutrients to plants [73] and increased soil aggregation [74]. Exploring and exploiting the potential of soil microbes, viz., rhizobia, PGPRs, and AMF, and their discrete or combinatorial application in soil can be an effective approach in increasing plant yield, ergo curtailing the inputs of conventional fertilizers, and consolidating their stress tolerance mechanisms [41]. A huge body of literature serves to illustrate the favorable influence of dual/triple inoculation on plant growth and tolerance under abiotic stress [75–78].

A very significant component of mycorrhizal technology is a dialogue with stakeholders and policymakers by means of workshops, advertisements, montages, awareness campaigns, or data-driven applications. Development of user-friendly apps would provide farmers with site-specific information and better aware them about the management practices and best AMF combinations to be used depending on the soil type, geography, climate, and crop system. The app will also provide suggestions to the farmers on the basis of input and would serve as a nexus among farmers, consultants, and consumers [67].

1.3.2 Commercialization of AM Technology: Mycorrhiza-Based Market and Products

Mycorrhizal industry has leaped toward the industrial stage on account of exhaustive research and commercial applications, foregrounding the ecological, economical, and sustainable aspects of the employment of AM [68, 79]. Multifarious benefits of AM have capacitated their commercial application. That being said, the market volume of AM has bloomed considerably during past two decades. Since the 1990s, there has been an exponential surge in the number of companies selling AM-based products. On a global level, the key contributors are located in Europe, Asia, North America, and Latin America [80]. India, followed by China, dominates the mycorrhiza-based market. Some of the major Indian manufacturers and sellers of mycorrhizal products are presented in Table 1.2.

The Indian market has witnessed a dramatic explosion during the last 10 years, possibly because of promotion of usage of biostimulants by the Government of India and one of the prominent research institutes working on mycorrhizal biology, propagation, and technology – The Energy and Resources Institute (TERI) [81]. Indian government is taking initiatives to call forth the deviation from conventional farming to organic, low-input farming inputs. In comparison with bigger business firms, from the USA, Germany, Italy, the UK, Spain, and Canada that export their

Table 1.2 Manufacturers and sellers of mycorrhiza-based products across India

Company/Institute	Headquarter	Trade name	Form of AM propagules
TERI	New Delhi	Ecorhiza-VAM/Nurserrhiza-VAM	Powder
Biotrack Technology Pvt. Ltd	Chennai, Tamil Nadu	RHIZAgold	Granular form
Safex Bio-Organics	Anand	VAM grow	Powder/ granules
Alex Bio Chem	Delhi	Natures Root	Powder
KCP Sugar and Industries Corporation Ltd.	Andhra Pradesh	Mycorrhiza VAM	Granules, liquid
Cadila Pharmaceuticals, Ltd	Ahmedabad	Josh	Granules, liquid
Cosme Biotech	Goa	Shubhodaya	Powder/ granules
Marutham Bio AgeS Innovations P Ltd.	Coimbatore, Tamil Nadu	VAM-101	VAM tablets
ManiDharma Biotech Pvt. Ltd.	Chennai, Tamil Nadu	ManiDharma VAM	Granules, liquid
Neologie Bio Innovations	Ahmedabad, Gujarat	Mycorrhiza VAM technical granules (HDPE bag) and VAM liquid	Granules, liquid
Ambika Biotech	Bhopal, Madhya Pradesh	Root Care	Powder
Jaipur Biofertilisers	Jaipur, Rajasthan	MycoGold	Powder
Dr. Rajan Laboratories	Tamil Nadu	Mycorrhizas	Powder, liquid, granules
TARI biotech	Thanjavur, Tamil Nadu	Vesicular-arbuscular mycorrhiza	VAM solution
Jaiveek Food and Fertilisers	Ahmedabad, Gujarat	JFF VAM	Powder, liquid
Sundaram Overseas Operation	Surat, Gujarat	Vesicular-arbuscular mycorrhiza (VAM)	Tablets/ liquid/ granular
T. Stanes and Company Limited	Coimbatore, Tamil Nadu	Solid formulations of arbuscular mycorrhiza	Powder/ granules
Neesa Agritech Private Limited	Ahmedabad, Gujarat	Arbuscular mycorrhiza	Powder and tablets
Majestic Agronomics Pvt. Ltd.	Una, Himachal Pradesh	Arbuscular mycorrhiza	Liquid
Birmon Biofertilizers Corporation	Delhi	EmraldBio	Liquid
Krishidhan Seeds Pvt. Ltd.	Dwarka, Delhi	Arbuscular mycorrhiza	Powder

(continued)

Table 1.2 (continued)

Company/Institute	Headquarter	Trade name	Form of AM propagules
Greenmax Agrotech	Coimbatore, Tamil Nadu	Gmax VAM	Powder
AgriLife	Hyderabad, Telangana	AgriVAM® *Rhizophagus irregularis*	Powder, liquid, granules

mycorrhizal products across geographical borders, the AM business is still a marginal enterprise for the local or regional markets [80]. Asia Pacific is the largest mycorrhiza-based biofertilizer market, with the European market being the fastest-growing and leading market for biostimulants. As projected in a report of the year 2019, AM-based market is valued at 268.8 million USD and is estimated to escalate to USD 621.6 million in 2025; the market is expected to experience a healthy growth rate in the years ahead. On a global scale, five major companies dominate the AM-based biofertilizer market, namely, Agri Life (UK), Agrinos (USA), Symborg (Spain), Sutane Natural Fertiliser (USA), and Valent BioScience LLC (USA) [82]. The major domains of application of commercial AM include horticulture, agriculture, gardening, forestry, landscaping, revegetation of degraded land, roof plantings, mycoremediation, and research [80].

Sophisticated understanding on AM symbiosis has glorified the commercial perspective for implementation of AM technology in various domains. Notwithstanding colossal potential of AM in agriculture, these are consciously introduced in a handful of industries [83], and the complete remunerative use of AM technology has yet to prosper. The progressive globalization and breakthrough technologies in conjunction with financial pressures are prejudicial to the dynamic biotechnology environment, which has further impacted the mycorrhizal industry. In developing countries, like India, AM technology will be the cynosure of all industries as the demand for low-input, organic food will increase. However, the process of transferring this proposition into a successful venture is facing blockades due to insufficient knowledge dissemination and lack of consultancy services. Consequently, mycorrhizal technology has landed up in reappropriation of technology or intellectual property [84].

1.3.3 Methodologies for Large-Scale Production of AMF

Mycorrhizal technology offers prospective benefits to small and marginal farmers who stand in dire need of economically feasible (low-cost) inputs to accelerate productivity of crops of high economic value (cereals, legumes, and horticultural crops, among others) [85]. Taking into account the immediate need for economically sustainable agriculture, dearth of inocula is a major roadblock for large-scale production of pure, pathogen-free, and highly infective AMF. Cultivation of AMF under axenic conditions is placed at the cutting edge of the headway of knowledge

on the use of biofertilizers. Various techniques have been proposed that can be used for bulk production of AMF on commercial scale, namely, nutrient film technique (NFT), aeroponics, root organ culture (substrate-free), and trap culture method (substrate-based). The quality and quantity of inoculum produced by the aforementioned systems are variable and consequently reflect on the net production cost [85].

Substrate-free techniques: Nutrient film technique, alias circulating hydroponic culture technique, can be used for rigorous production of AMF inocula but under stringently defined conditions. In NFT, the plant roots remain immersed in a thin layer of nutrient solution (i.e., film) flowing inside an inclined channel where plant roots and AMF fungus develop [85, 86]. Continuous optimization and maintenance of pH and nutrient concentration is ensured in the flowing nutrient solution [87]. The solid mat of plant roots that develop inside the gulls (NFT channels) are ideal for the production of concentrated, voluminous, and easily harvestable form of inoculum than produced by substrate- based methods (trap culturing in soil/sand/expanded clay). In aeroponics, on the other hand, the plant roots are suspended in nutrient mist rather than in flowing/static nutrient solution or in solid media [88]. Increased aeration of culture media is achieved by spraying of microdroplets, and gas exchange is permitted by liquid film that surrounds the roots growing therein [86]. Ultrasonic nebulizer technology in aeroponics (that ejects microdroplets of 1 μm in diameter) is deemed as the most appropriate aeroponics method for *G. intraradices* cultivation using Sudan grass [89]. In substrate-free inoculum development systems (NFT and aeroponics), pre-colonized plants are introduced inside the system. Plant seedlings and AMF propagules are precultured in appropriate substrate in pots for several weeks.

Employment of root organ culture (ROC) is a good alternative for anexic production and propagation of AMF inoculum. Preliminary attempts to culture AMF in vitro date back to the 1950s. Since then, ROC has been used rigorously to study the biological function of AMF and also for bulk production of pathogen-free AMF inoculum. Mechanistically, plant roots are transformed by T-DNA of Ri plasmid of *Agrobacterium rhizogenes*, and the pathogen condition thus induced is known as hairy root [90]. These hairy roots are biochemically and genetically fixed and can be propagated in hormone-free media [91]. ROC is a significant tool to understand the biology of AMF and achieve bulk production of its monoxenic inocula [92]. Several *Glomus* species (e.g., *G. microcarpum*, *G. intraradices*, *G. mosseae*, *G. fasciculatum*, and *G. margarita*) have been successfully used to set up substrate-free inoculum production [[92–95]; Table 1.3].

Each of these systems has associated pros and cons. Aeroponics technique offers to be a good technique in terms of rapid and profuse sporulation and root colonization owing to the highly aerated rooting environment. With both aeroponics and ROC, high-quality monoxenic culture is produced, whereas chances of contamination through NFT are very high. Moreover, complete immersion of root system into the media dramatically reduces the oxygen availability and establishes a zone of "nutrient-poor" water around roots. The major challenge affiliated to NFT and aeroponics is extra maintenance in terms of several major aspects, viz., nutrient concentration, temperature, pH, gaseous atmosphere, droplet size, and mist cycle

Table 1.3 AMF propagule production in substrate-free cultivation system

Fungus species	Host plant	Technique used	Reference
Glomus mosseae	*Zea mays*	Nutrient film technique	[96]
G. fistulosum	*Fragaria x ananassa*	Hairy roots	[97]
Glomus mosseae	*Triticum aestivum*, *Linum usitatissimum*, *and Sorghum bicolor*	Hydroponics	[93]
Glomus deserticola, Glomus etunicatum, G. intraradices	*Ipomoea batata*	Aeroponics	[98]
Glomus etunicatum	*Paspalum notatum*		
Glomus intraradices	*L. usitatissimum*	Hydroponics	[99]
Glomus sp. *(INVAM-FL329)*	*Paspalum notatum Allium cepa*	Hydroponics	[100]
Glomus intraradices	*Sorghum sudanense*	Aeroponics	[89]
Glomus intraradices	*Phaseolus vulgaris*	Hydroponics	[95]
Gigaspora margarita and Glomus intraradices	*Daucus carota*	Hairy root culture	[101]
G. simosum	*D. carota*	Root organ culture	[102]
G. mosseae	*Lactuca sativa*	NFT	[94]
Glomus microcarpum	*Vigna vexillata*	Ri-TDNA transformed hairy roots	[103]
	Ipomoea batata		[104]
Rhizophagus irregularis	*D. carota*	Hairy root culture	[105]

(for aeroponics). When all aspects are taken into consideration, ROC offers apparent advantages over other systems, allowing the observation of AMF development and morphology in vitro [85] while allowing AMF to finish their life cycle [106]. The edge of ROC over the other two techniques comes from the fact that physicochemical and biological parameters of the system can be conveniently controlled and the collection and storage of inoculum is also easy in petriplates [85].

Substrate-based technique: Conventional production of AMF inocula is carried out by cultivation of host plants and associated mycobiont in a soil-/sand-based substrate. The substrate-based production is often performed under optimized conditions of light, temperature, and humidity in greenhouse or growth chambers. Based on the prevailing climate and host plant, large-scale production is achieved in open air on field plots [86, 107]. The mother inoculum used in mass production is mostly a single known AMF species (monoculture) or a consortium of known selected AMF species, while on-farm (open air) production is mostly started with AMF species indigenous to that site [107]. The initiator inoculum constitutes a mixture of spores, chopped and dried mycorrhized roots of the host, as well as the soil containing the AMF spores and hypha [108]. A wide range of plant hosts, namely, *Zea mays*, *Allium* spp., *Coleus* spp., *Paspalum notatum*, *Trigonella foenum*

graecum, *Trifolium* spp., *Medicago sativa*, and *Sorghum vulgare*, among others, are frequently used for mass production of AMF. The choice of host plants used for propagation of AMF species is based on multiple aspects, the trap plants must preferably have a short life cycle, robust root system, tolerance to low phosphorus levels, temperature extremities, and pathogenic infection, and should possess discernable features in colonized vs. uncolonized roots, as in case of maize and leek, colonized roots develop yellow appearance as opposed to uncolonized white roots [109].

One of the important determinants for inoculum production is host-dependent sporulation of AMF species. Gaur and Adholeya [107] inferred from their study that the degree of propagule production depends on the host species. High intraradical colonization percentage is important for the achievement of a mixed spore-root inoculum, which might not be a complete necessity for production of spore inoculum. Thus, the choice of inoculum partially determines the host plant and associated fungus chosen [86].

Various substrates such as peat, glass beads, compost, perlite, and vermiculite or calcinated clay are also used for conventional production of AMF inoculum [94, 110–112]. Vermiculite and perlite (comparatively inert carriers) are used to adulterate nutrient-rich compost/soil [111]. In reverse, nutrient-rich organic substrates (e.g., peat) can be used to fertilize nutrient-deficient soil. The addition of glass beads or river sand (amendments) helps in facilitating easy harvest and cleanup of fungal mycelia from debris [110]. The dimension of substrate particles is an important regulator of aeration, moisture content, and drainage (porosity) thereof. These parameters have been found to, in turn, influence the degree of sporulation of AMF [107]. Furthermore, alteration of nutrient regimes has also been shown to influence the production of AMF propagules [111]. Generally, low-nutrient conditions (low phosphorus) are deemed favorable for AMF colonization. Guar and Adholeya [108] reported increased production of *G. intraradices* grown in association with maize in sand substratum amended with nutrient solution devoid of phosphorus. Likewise, amendment of sand-based system with phosphorus concentration (>2 μM) ensued in poor sporulation levels in AMF species (*Glomus mosseae*, *G. etunicatum*, and *Gigaspora margarita*). Several other additional factors, such as pH, cation exchange capacity, light intensity, temperature, water content, and carbon allocation to roots, majorly influence the production of AMF propagules [86].

Substrate-based production of AMF is an extensively adopted technique owing to its cost-economics, i.e., cheap consumables and low labor and technical input. This system facilitates the production of dense masses of discrete or consolidated propagules of different species, as much as 80–100 propagules/cm^3 [113]. However, this production system does not guarantee a contaminant-free output, nevertheless stringent quality control measures. In addition to that, these require large spaces and regulatory measures to keep pest and pathogen infiltration at abeyance [86]. As opposed to substrate-free production methods, the inoculum produced from substrate-based systems is not befitting for direct mechanical application [114].

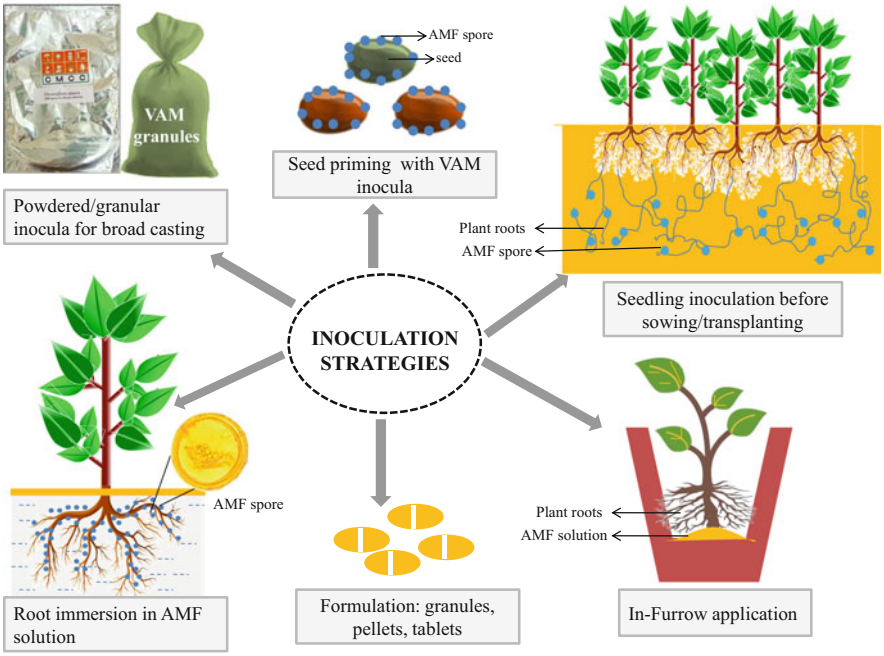

Fig. 1.1 Several application technologies and inoculation strategies of AMF inocula

Post bulk production of fungal propagules, their formulation should be done in a way to ensure storage and distribution under a wide range of temperatures under guaranteed period, and subsequently their easy transport and application. For successful colonization to result, AMF propagules should be in close proximity to the plant roots (Fig. 1.1). Inoculum formulation may also contain other helper organisms, such as MHBs or fungi, which cooperatively enhance desirable traits in the candidate plants. Majorly, there are five application technologies for AMF, namely, broadcasting, in-furrow application, seed dressing, root-dipping, and seedling inoculation [83] (Fig. 1.1) However, the nature of final formulation may vary from a dry formulation to gel formulation or non-sterile liquid media [79].

1.3.4 Constraints in Mycorrhizal Technology

The expediency of commercializing AMF inocula has been contingent on the ease and economics of extensive production and formulation of voluminous amounts of viable and efficacious mycorrhizal propagules with higher shelf life [83]. Primarily, large-scale production and in vitro propagation of AMF is limited by its obligate symbiotic nature; as established earlier, growth of AMF is completely host-dependent. Despite explosion of studies on benefits of mycorrhiza and flourishing

enterprises vested in production of AMF inocula, the full potential of the current "mycorrhizal market" remains untapped. Other than technical challenges, the three major roadblocks for commercialization of AMF products include (i) quality assurance of the inoculum, (ii) political constraints, and (iii) acceptance from consumers (farmers) [80]. The most premium way to warrant the quality of inocula produced is by ROC in sterile conditions; however, not all fungal species can be successfully propagated under in vitro anexic conditions. Quality production is a necessary requirement for basic research and application of AMF propagules [86]. Consequently, invasive microbes might get inadvertently introduced into the fields [115]. Although no invasive AMF strain has been reported thus far, introduction of non-native species might be restrained by the indigenous AMF strains [86]. With regard to product quality, lack of regulatory bodies to put forth standard quality parameters has resulted in quality standards being "self-imposed" by the companies (manufacturers of AMF-based material) to ensure their best production [4], and those parameters might not be adequate enough for quality assurance of the inocula produced.

Another problem of inoculation is the lack of consistent favorable results in the fields. At times, the abundance of native fungi in the target cultivation system is so much so that they can be adequately efficacious and cost of inoculation (purchase and labor) is not offset by the outcome. Another setback associated with mycorrhizal application is the intervention of agricultural practices, such as fertilizer/pesticide amendments, cropping patterns, tillage, and long periods of fallow, which hamper mycorrhizal association and development, more so on upper layers of soil [24]. Mycorrhizal development has been reported to be prejudiced by the extensive use of systemic fungicides and insecticides especially by the copper-based formulations of these chemicals [116]. Besides, the allelopathic effect of AMF through common mycorrhizal network (CMN) also accounts to the challenges affiliated with AMF technology [117]. On this front, more research trajectories should be directed toward exploring the implication of inoculum introduction in agroecosystems on allelochemical alterations, since CMN serves as "high ways" for the movement of secondary metabolites from donor to the candidate plant [79].

Mass production of AMF inoculum is a multiplex procedure involving necessary biotechnological expertise, as well as responsiveness to legal, cultural, ethical, education, and commercial requirements. Not all mycorrhizal products are authentic; many of them are "snake oils" that do not meet the promised deliverables of the product. Such products are falsely labeled as mycorrhizal inoculum; however, the actual contents are just quack remedies with very low or negligible quantity of AMF propagules [79]. The available over-the-counter AMF-based products differ in major degrees with respect to the number and abundance of species claimed to be present, number and volume of spores, and the nature of carrier media. The escalating market of spurious organic−/bio-based products and lack of strict quality checkpoints result in the delivery of bogus AMF products that can potentially impact the health of plants and natural resources [84]. Unfortunately, people at the receiving end of the inoculum, i.e., farmers and horticulturists, have limited or no provisions to test the efficacy or estimate the mechanism of these products before purchasing [79].

Finally, not every plant-AMF combination is equally beneficial and effective; hence, it is preferable to screen different AMF strains for each crop to confirm the most favorable plant-AMF combination. Assembling the befitting consortia of host plant phenotype and soil microbiota is an underground revolution that will strive for ecological amplification in agriculture [118]. The retail and wholesale segment of mycorrhizal market has seen a remarkable outburst all through the last two decades. The clientele range is quite broad, starting from local gardeners and commercial growers to public and private institutions [101]. Much accentuation on organic farming and sustainable agriculture coupled with the concrete evidence of the encouraging effect of AMF on overall health, productivity, and fitness of crop plants has been consequential in bringing about the observed growth in AMF technology and industries. Economic feasibility of this technology accounts for its increasing prominence and appraisal as a sustainable strategy of crop production in the current layout of climate-resilient agri-technology [84].

1.4 Economic Evaluation of AM Symbiosis

Time and again, the symbiotic association of AMF and plants has been deliberated referring to plant growth and yield, as they positively influence plants' nutrition, yield, and quality of products, protect plants from a/biotic stresses, and provide other benefits such as soil structure improvement [21, 119, 120]. Despite the widely accepted fact that AMF play a vital part in attaining sustainable agriculture, a "state of confusion" still exists among farmers and regulatory bodies for adopting this technology. This, in itself, presents as a reason for why there is a need for economic assessment of this symbiotic association. In addition, as economic valuation of AM symbiosis is seldom deliberated [111], examining its potential economic aspects in order to harness "mycorrhizal technology" is paramount. There are two schools of thought on the economic benefits of AMF. While one considers that the benefits imparted by AMF to farmers are exaggerated and extrapolated, the other counteracts by accentuating the indirect and non-tangible economic contributions of AMF such as their role in ecosystem functioning via promoting soil aggregation, reducing loss of nutrients/ nutrient leaching, and improving quality of yield and grain fortification [56, 67, 121, 122]. Hence, a detailed economic valuation of AM technology would increase its acceptability by farmers and policymakers worldwide [123]. Since AMF provide both quantitative and qualitative benefits, economic assessment becomes a bit difficult. It should be kept in mind that relying exclusively on monetary figures of cost and benefits would be misleading since this incomplete approach will not disclose their qualitative effects. As a result, economic assessment of this symbiosis should be carried out following both cost-benefit assessment method (CBM) used for the benefits imparted by measurable parameters and contingent assessment methods (CM) for the benefits imparted by nonmeasurable parameters (Fig. 1.2).

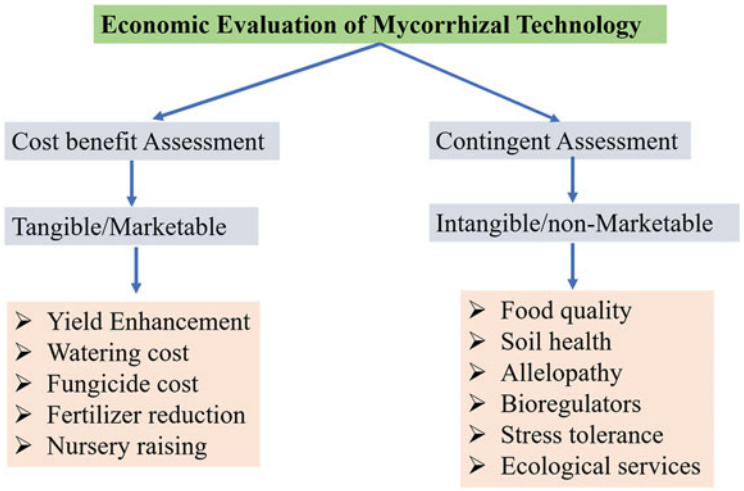

Fig. 1.2 Overview of the economic assessment methods of mycorrhizal technology

1.4.1 Assessment of the Tangible Benefits Provided by AMF

It is the most common form of economic analysis, often used to assess the net benefit of any technology/project [124]. For economic assessment of AMF through CBM, the parameters for which direct costing can be calculated are taken into consideration. The net result of the symbiosis is taken into account for measuring its "cost" or "benefit" where satisfactory net result is considered as "benefit" and the undesirable net result as "cost." The cost of mycorrhizal technology comes in the form of procurement and application of AMF inoculum (development of mycorrhizal technology). As discussed earlier in text, inocula can be produced using different approaches, i.e., substrate-based and substrate-free. Chen et al. [80] documented that the average cost of AMF inoculum production for semiprofessional users was approximately in the range of 10–50 cents (USD) per plant. Inoculum cost for commercial use in large agricultural scale was found to be relatively inexpensive [125, 126].

On the other hand, the monetary benefits of AMF can be estimated by quantifying the savings in expenses. For instance, it is well established that mycorrhizal fungi can improve plant nutrition, and this can be reproduced in the form of savings made in fertilizer application. Similarly, enhancement in crop yield and tolerance to a/biotic stresses brought by AMF in large scale can be converted into monetary assets in terms of insecticide/pesticide application costs and so on. It is worth keeping in mind that for such assessment, a hypothetical baseline, i.e., "absence of AMF," should be defined to evaluate the net impacts of the parameters considered. However, absence of AMF in open-field soil is rare, thereby posing difficulty in estimating contributions of AMF in field conditions [127]. A study on cost-benefit estimation for commercial production of *Rosa* spp. showed that AMF inoculation

during plantation reduced application of fertilizers to 50%, thereby reducing the expenses on fertilizers and saving approximately 267 Euros per hectare per month [79]. Nevertheless, other studies on crops such as cereals reported AMF application to be expensive or ineffective [128]. Although there is still a controversy regarding application of AMF in agricultural field at present, it is high time that the "concerns" come in agreement on the need for developing better methodologies to evaluate AMF's impact in agroecosystem, without which this potent natural resource might stay untapped [129, 130].

1.4.2 Assessment of the Non-tangible Benefits Provided by AMF

Contingent valuation is used to assess qualitative values or the nonmeasurable benefits of an organism such as AMF. Use of contingent valuation is important for complete assessment of mycorrhizal technology as AMF create social and environmental impacts, which cannot be valued and thus get overlooked. The non-tangible benefits provided by AMF include enhancement in food quality such as grain fortification, improving soil structure by promoting formation of soil aggregate, preventing nutrient leaching owing to higher water retention capacity, and so on [21, 56, 120, 122]. In the past, contingent assessment was done for valuation of urban farming, land degradation and restoration, and biodiversity [124, 132]. Till date, contingent assessment on AMF has not been carried out yet. To summarize the process as adopted by previous assessment of urban farming, biodiversity, and so on, CM involves asking questions to respondents (stakeholders) much like in a survey, and the answers provided were used to assign monetary value [124]. For instance, one may ask how much a stakeholder would be willing to pay to get the individual benefits of mycorrhiza or to avoid risks caused due to its absence. The initial step in contingent valuation is determination of the specific nature of what impacts need to be valued [124] followed by identification of affected stakeholders (farmers, food markets, resource owners, agriculture agencies, planning and land use agencies, and environmentalists). Identifying stakeholders is critical to complete the analysis and establish credibility. The third step involves conducting a survey with the aim to measure its impact on a scale. The participants are generally asked to use a common scale to designate values for the impacts. Question preparation is principally an imperative step to obtain information. In such way, assessment of non-tangible benefits of AMF can be done by determining the value attached by stakeholders. All values in the questionnaire are defined to a scale, either monetary or trade-off, and later converted to monetary scale to summarize the final value [124, 133]. As contingent valuation relies on people's response, controversy may arise in the integrity of value assigned to an item.

1.4.3 Constraints in Economic Valuation of Mycorrhizal Technology

The uncertain behavior of AMF under field conditions presents as a major limitation in encasing its benefits [134]. Their functioning in field condition depends upon various factors such as inoculation time, co-adaptability with partners, soil, agronomic, and climatic factors [53, 131, 135]. An approach to approve economic benefits of AMF under field conditions can be ascertaining a relation between occurrence of the fungus and the influence on yield or other ecosystem functions. Various methods adopted in evaluating the economic benefits of mycorrhizal technology under field conditions include assessment of different variables for mycorrhizal effects, which at times poses risks of wrong assessment. Some source of wrong assessment of AMF benefits and their potential management strategies include the following:

(i) Variation in the quality and function of the inoculum in field conditions. It has been reported that many fungal spores do not germinate in field [136]. As a result, the optimal density must be standardized taking into consideration the crop variety, soil type, and environmental condition.
(ii) Estimating the total contribution of AMF to plant yield is tough as non-colonized plants (that serve as control) in fields are not usually found [128]. For this, preliminary studies on modeled agroecosystems should be conducted.
(iii) The status of soil nutrient richness influences the occurrence and effectiveness of AMF. AMF inoculated in phosphorus-rich but nitrogen-limited soil reportedly showed the fungi taking up the role of parasitic fungi, thereby hindering growth and development of host plants [53].
(iv) Compatibility of host and AMF varies [137]. Resultantly, the responses to fungal colonization vary among genotypes of both host and fungal symbiont. An in-depth knowledge of this complex interaction is necessary [135]. Best performing combination must be selected for each situation.
(v) The interaction of AMF with the existing microflora of soil can be positive, negative, or even neutral. Hence, knowledge on such potential interaction will help in avoiding the risk of incorrect assessment of the mycorrhizal benefits.

1.5 Status Quo and Future Research/Avenues

Arbuscular mycorrhizal technology is a significantly beneficial and economically lucrative option than chemical fertilization and amendments. However, the translation of this technique to technology involves attenuation at several levels, determination of key rate-limiting factors, economically and environmentally safe inputs, and debottlenecking of production. Though AM technology is a straightaway option to intensify the productivity of ecosystem (both managed and natural), it encounters many constraints in its commercialization, not least in relation to the lack of

multilocation wide-ranging field trials under different ecological conditions and reluctance of end users to shift from energy-intensive system to an energy-conservative system due to quality apprehensions of cultivators about available AMF inocula from varied sources [83]. With regard to this, legislative intervention coupled with promotion of this technology by influential bodies like IMS (International Mycorrhizal Society) is desirable to amplify the global acceptance of these biostimulants [80].

Advocation and literacy drives on the promiscuous relation of plant-AMF could be effectual in unfurling new avenues and approaches for mycorrhizal industries so as to promote this technology in sustainable agriculture systems, guaranteeing the availability of quality product in the market. For commercialization of AM technology to be completely accomplished, it should be efficient and outstanding in outcomes. Constructing an entrepreneurial culture affirmed by solid research framework, network, and funding is another essential requirement [84]. Further, collaboration between private firms and scientific community can be consequential as the development of the former can propel further research, which in turn will broaden the application and maturation of mycorrhizal industry [4].

Though AM technology is a promising approach, it is associated with intrinsic risks. The benefits offered by AMF inoculum are highly context-dependent, and administration of exotic genotype always bears the likelihood of environmental impacts thereof [53, 138]. The variability obtained in results makes the credence of agricultural community in the generability and efficiency of AMF precarious. Thus, AMF inoculum should not be used indiscriminately without proper knowledge of plant phenotype, rhizosphere microbiome, and community constitution of native AMF across different rooting depths. In view of the foregoing, more fundamental, descriptive research (both molecular and spore-based), it is needed to understand the vertical distribution of AMF in agricultural fields across the entire soil profile [139]. Regardless of three-dimensional nature of soil, the focus of researchers has mostly been centered on its shallow layers with virtually little or no consideration for the ecosystem contributions of AMF species from deeper realms of soil [140]. In this context, DNA barcoding of AMF, as promoted by Kruger et al. [141], and Stockinger et al. [142], provides the baseline of AMF traceability within the fields. Furthermore, crop breeding programs targeting the traits of mycorrhizal responsiveness are needed to leverage the benefits of symbiosis in crop production. Likewise, "crossing" of AMF (by anastomosis) and its subsequent culturing can produce genotypes with novel symbiotic features. Since anastomosis can happen only between compatible AMF isolates, the fungus can be bred for refined symbiotic characters by genetic intermixing between distant (but compatible) strains. This should be followed with screening of new strains with host plant of interest, facilitating the identification of best AMF segregant for each species of host [143].

1.6 Conclusions

Arbuscular mycorrhizal fungi occur in nature as community of organisms involved in a network of biotic interactions within the soil, dynamically responding to a range of environmental signals and management factors. This multiplex interaction of AMF in soil is quintessential complex ecological and evolutionary situation that rebels "one-size-fits-all" solution [67]. Thus, appropriate development of technology inclusive of all five components is coveted, with consideration for social sustainability and balanced economy, matching the complexity of plant-AMF interactions to better exploit the mycorrhizal symbiosis for achieving sustainable agroecosystems. Taken together, a better acquaintance of the relative contribution of AMF to any aspect of sustainability, cognizance of parameters influencing mycorrhizal effectiveness, and dilation of response variables for inventorizing mycorrhizal effects are major research needs that are crucial to reinforce the mycorrhizal technology.

References

1. Remy W, Taylor TN, Hass H, Kerp H (1994) Four hundred-million-year-old vesicular arbuscular mycorrhizae. Proc Natl Acad Sci U S A 91:11841–11843
2. Basu S, Rabara RC, Negi S (2018) AMF: the future prospect for sustainable agriculture. Physiol Mol Plant Path 102:36–45
3. Basiru S, Mwanza HP, Hijri M (2021) Analysis of arbuscular mycorrhizal fungal inoculant benchmarks. Microorganisms 9:81–99
4. Vosátka M, Albrechtová J, Patten R (2008) The international market development for mycorrhizal technology. In: Varma A (ed) Mycorrhiza. Springer, Berlin, Heidelberg, pp 419–438
5. Berruti A, Lumini E, Balestrini R, Bianciotto V (2016) Arbuscular mycorrhizal fungi as natural biofertilizers: let's benefit from past successes. Front Microbiol 6:1559–15572
6. Babalola OO (2010) Beneficial bacteria of agricultural importance. Biotechnol Lett 32:1559–1570
7. Alori ET, Glick BR, Babalola OO (2017) Microbial phosphorus solubilization and its potential for use in sustainable agriculture. Front Microbiol 8:971–984
8. Sreenivasa MN, Bagyaraj DJ (1989) Use of pesticides for mass production of vesicular-arbuscular mycorrhizal inoculum. Plant Soil 119:127–132
9. Abbasi H, Akhtar A, Sharf R (2015) Vesicular arbuscular mycorrhizal (VAM) fungi: a tool for sustainable agriculture. Am J Plant Nutr Fertilization Technol 5:40–49
10. Aradottir AL, Hagen D (2013) Ecological restoration: approaches and impacts on vegetation, soils and society. Adv Agron 120:173–222
11. Jacobs DF, Oliet JA, Aronson J, Bolte A, Bullock JM et al (2015) Restoring forests: what constitutes success in the twenty-first century? New For 46:601–614
12. CBD (2010) Aichi Biodiversity Targets. http://www.cbd.int/sp/targets/
13. Birhane E, Sterck FJ, Fetene M, Bongers F, Kuyper TW (2012) Arbuscular mycorrhizal fungi enhance photosynthesis, water use efficiency, and growth of frankincense seedlings under pulsed water availability conditions. Oecol 169:895–904
14. Manaut N, Sanguin H, Ouahmane L, Bressan M, Thioulouse J, Baudoin E et al (2015) Potentialities of ecological engineering strategy based on native arbuscular mycorrhizal community for improving afforestation programs with carob trees in degraded environments. Ecol Eng 79:113–119

15. Augé RM (2004) Arbuscular mycorrhizae and soil/plant water relations. Can J Soil Sci 84: 373–381
16. Cavagnaro TR, Smith FA, Smith SE, Jakobsen I (2005) Functional diversity in arbuscular mycorrhizas: exploitation of soil patches with different phosphate enrichment differs among fungal species. Plant Cell Environ 28:642–650
17. Wilson GW, Rice CW, Rillig MC, Springer A, Hartnett DC (2009) Soil aggregation and carbon sequestration are tightly correlated with the abundance of arbuscular mycorrhizal fungi: results from long-term field experiments. Ecol Lett 12:452–461
18. Rillig MC, Wright SF, Shaw MR, Field CB (2002a) Artificial climate warming positively affects arbuscular mycorrhizae but decreases soil aggregate water stability in an annual grassland. Oikos 97:52–58
19. Leake J, Johnson D, Donnelly D, Muckle G, Boddy L, Read D (2004) Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. Can J Bot 82:1016–1045
20. Bedini S, Turrini A, Rigo C, Argese E, Giovannetti M (2010) Molecular characterization and glomalin production of arbuscular mycorrhizal fungi colonizing a heavy metal polluted ash disposal island, downtown Venice. Soil Biol Biochem 42:758–765
21. Rillig MC (2004) Arbuscular mycorrhizae, glomalin, and soil aggregation. Can J Soil Sci 84: 355–363
22. Rillig MC, Mummey DL (2006) Mycorrhizas and soil structure. New Phytol 171:41–53
23. Soka G, Ritchie M (2014) Arbuscular mycorrhizal symbiosis and ecosystem processes: prospects for future research in tropical soils. Open J Ecol 4:11–22
24. Oehl F, Sieverding E, Ineichen K, Ris EA, Boller T, Wiemken A (2005) Community structure of arbuscular mycorrhizal fungi at different soil depths in extensively and intensively managed agroecosystems. New Phytol 165:273–283
25. Görlach B, Landgrebe-Trinkunaite R, Interwies E, Bouzit M, Darmendrail D & Rinaudo JD (2004) Assessing the economic impacts of soil degradation. Volume IV: Executive Summary. Study commissioned by the European Commission, DG Environment, Berlin
26. Janos DP (1980) Mycorrhizae influence tropical succession. Biotropica:56–64
27. Renker C, Zobel M, Öpik M, Allen MF, Allen EB et al (2004) Structure, dynamics, and restoration of plant communities: do arbuscular mycorrhizae matter. Assembly rules and restoration ecology: bridging the gap between theory and practice, vol 5. Island Press, Washington, p 189
28. Kikvidze Z, Armas C, Fukuda K, Martínez-García LB, Miyata M et al (2010) The role of arbuscular mycorrhizae in primary succession: differences and similarities across habitats. Web Ecol 10:50–57
29. Cripps CL, Edding-ton LH (2005) Distribution of mycorrhizal types among alpine vascular plant families on the Beartooth Plateau, Rocky Mountains, USA, in reference to large-scale patterns in arctic-alpine habitats. Arct Antarct Alp Res 37:177–188
30. Song YY, Zeng RS, Xu JF, Li J, Shen X et al (2010) Interplant communication of tomato plants through underground common mycorrhizal networks. PLoS One 5:13324
31. Smith SE, Read DJ (2010) Mycorrhizal symbiosis. Academic press, San Diego, p 800
32. Rillig MC, Wright SF, Eviner VT (2002b) The role of arbuscular mycorrhizal fungi and glomalin in soil aggregation: comparing effects of five plant species. Plant Soil 238:325–333
33. Singh PK (2012) Role of glomalin related soil protein produced by arbuscular mycorrhizal fungi: a review. Agric Sci Res J 2:119–125
34. Singh PK, Singh M, Tripathi BN (2013) Glomalin: an arbuscular mycorrhizal fungal soil protein. Protoplasma 250:663–669
35. Brito I, Goss MJ, Alho L, Brígido C, van Tuinen D, Félix et al (2019) Agronomic management of AMF functional diversity to overcome biotic and abiotic stresses-the role of plant sequence and intact extraradical mycelium. Fungal Ecol 40:72–81
36. Dowarah B, Gill SS, Agarwala N (2021) Arbuscular mycorrhizal fungi in conferring tolerance to biotic stresses in plants. J Plant Growth Regul:1–16

37. Begum N, Qin C, Ahanger MA, Raza S, Khan MI et al (2019) Role of arbuscular mycorrhizal fungi in plant growth regulation: implications in abiotic stress tolerance. Front Plant Sci 10: 1068
38. Devi TS, Gupta S, Kapoor R (2019) Arbuscular mycorrhizal fungi in alleviation of cold stress in plants. In: Satyanarayana T, Deshmukh SK, Deshpande MV (eds) Advancing frontiers in mycology & mycotechnology. Springer Nature, Singapore, pp 435–455
39. Evelin H, Devi TS, Gupta S, Kapoor R (2019) Mitigation of salinity stress in plants by arbuscular mycorrhizal symbiosis: current understanding and new challenges. Front Plant Sci 10:470
40. Gupta S, Thokchom SD, Kapoor R (2021) Arbuscular mycorrhiza improves photosynthesis and restores alteration in sugar metabolism in *Triticum aestivum* L. grown in arsenic contaminated soil. Front Plant Sci 12:334
41. Sharma K, Gupta S, Thokchom SD, Jangir P, Kapoor R (2021) Arbuscular mycorrhiza-mediated regulation of polyamines and aquaporins during abiotic stress: deep insights on the recondite players. Front Plant Sci 12:1072
42. Mandal S, Evelin H, Giri B, Singh VP, Kapoor R (2013) Arbuscular mycorrhiza enhances the production of stevioside and rebaudioside-A in *Stevia rebaudiana* via nutritional and non-nutritional mechanisms. Appl Soil Ecol 72:187–194
43. Kapoor R, Giri B, Mukerji KG (2004) Improved growth and essential oil yield and quality in Foeniculum vulgare mill on mycorrhizal inoculation supplemented with P-fertilizer. Bioresour Technol 93(3):307–311
44. Mandal S, Upadhyay S, Singh VP, Kapoor R (2015) Enhanced production of steviol glycosides in mycorrhizal plants: a concerted effect of arbuscular mycorrhizal symbiosis on transcription of biosynthetic genes. Plant Physiol Biochem 89:100–106
45. Thokchom SD, Gupta S, Kapoor R (2020) Arbuscular mycorrhiza augments essential oil composition and antioxidant properties of *Ocimum tenuiflorum* L.–A popular green tea additive. Ind Crop Prod 153:112–418
46. Brundrett MC & Abbott LK (2002). Arbuscular mycorrhizas in plant communities. In Microorganisms in plant conservation and biodiversity (pp. 151–193). Springer, Dordrecht
47. Herman DJ, Firestone MK, Nuccio E, Hodge A (2012) Interactions between an arbuscular mycorrhizal fungus and a soil microbial community mediating litter decomposition. FEMS Microbiol Ecol 80:236–247
48. Soka G, Ritchie M (2014) Arbuscular mycorrhizal symbiosis and ecosystem processes: prospects for future research in tropical soils. Open J Ecol:2014
49. Osorio NW, Habte M (2001) Synergistic influence of an arbuscular mycorrhizal fungus and a P solubilizing fungus on growth and P uptake of *Leucaena leucocephala* in an Oxisol. Arid Land Res Manag 15:263–274
50. Parniske M (2008) Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat Rev Microbiol 6:763–775
51. Banerjee K, Gadani MH, Srivastava KK, Verma N, Jasrai YT et al (2013) Screening of efficient arbuscular mycorrhizal fungi for *Azadirachta indica* under nursery condition: a step towards afforestation of semi-arid region of Western India. Brazilian J Microbiol 44:587–594
52. Barea JM, Palenzuela J, Cornejo P, Sánchez-Castro I, Navarro-Fernández C et al (2011) Ecological and functional roles of mycorrhizas in semi-arid ecosystems of Southeast Spain. J Arid Environ 75:1292–1301
53. Hoeksema JD, Chaudhary VB, Gehring CA, Johnson NC, Karst J et al (2010) A meta-analysis of context-dependency in plant response to inoculation with mycorrhizal fungi. Ecol Lett 13: 394–407
54. Smith SE, Smith FA (2011) Roles of arbuscular mycorrhizas in plant nutrition and growth: new paradigms from cellular to ecosystem scales. Annu Rev Plant Biol 62:227–250
55. Smith FA, Jakobsen I, Smith SE (2000) Spatial differences in acquisition of soil phosphate between two arbuscular mycorrhizal fungi in symbiosis with *Medicago truncatula*. New Phytol 147:357–366

56. Cavagnaro TR, Bender SF, Asghari HR, van der Heijden MG (2015) The role of arbuscular mycorrhizas in reducing soil nutrient loss. Trends Plant Sci 20:283–290
57. Köhl L, van der Heijden MG (2016) Arbuscular mycorrhizal fungal species differ in their effect on nutrient leaching. Soil Biol Biochem 94:191–199
58. Van Der Heijden MG (2010) Mycorrhizal fungi reduce nutrient loss from model grassland ecosystems. Ecology 91:1163–1171
59. Nouri E, Breuillin-Sessoms F, Feller U, Reinhardt D (2014) Phosphorus and nitrogen regulate arbuscular mycorrhizal symbiosis in *Petunia hybrida*. PLoS One 9:e90841
60. Evelin H, Kapoor R, Giri B (2009) Arbuscular mycorrhizal fungi in alleviation of salt stress: a review. Ann Bot 104:1263–1280
61. http://www.un.org/esa/sustdev/documents/agenda21/french/action12.htm
62. Kubikova E, Jennifer LM, Bonnie HO, Michael DM, Augé MR (2001) Mycorrhizal impact on osmotic adjustment in *Ocimum basilicum* during a lethal drying episode. J Plant Physiol 158: 1227–1230
63. de Souza Moreira F M, Ferreira PAA, Vilela LAF & Carneiro MAC (2015). Symbioses of plants with rhizobia and mycorrhizal fungi in heavy metal-contaminated tropical soils. In Heavy Metal Contamination of Soils (pp. 215–243). Springer, Cham
64. Punamiya P, Datta R, Sarkar D, Barber S, Patel M, Das P (2010) Symbiotic role of *Glomus mosseae* in phytoextraction of lead in vetiver grass [*Chrysopogon zizanioides* (L.)]. J Hazard Mater 177:465–474
65. Audet P (2014). Arbuscular mycorrhizal fungi and metal phytoremediation: ecophysiological complementarity in relation to environmental stress. In Emerging technologies and management of crop stress tolerance (pp. 133–160). Academic Press
66. Kanwal S, Bano A, Malik RN (2015) Effects of arbuscular mycorrhizal fungi on wheat growth, physiology, nutrition and cadmium uptake under increasing cadmium stress. IJAAR 7:30–42
67. Rillig MC, Sosa-Hernández MA, Roy J, Aguilar-Trigueros CA, Vályi K et al (2016) Towards an integrated mycorrhizal technology: harnessing mycorrhiza for sustainable intensification in agriculture. Front Plant Sci 7:1625
68. Gianinazzi S, Gollotte A, Binet MN, van Tuinen D, Redecker D et al (2010) Agroecology: the key role of arbuscular mycorrhizas in ecosystem services. Mycorrhiza 20:519–530
69. Johnson NC (2010) Resource stoichiometry elucidates the structure and function of arbuscular mycorrhizas across scales. New Phytol 185:631–647
70. Chanway CP, Holl FB (1991) Biomass increase and associative nitrogen fixation of mycorrhizal *Pinus contorta* seedlings inoculated with a plant growth promoting *Bacillus* strain. Can J Bot 69:507–511
71. Meyer JR, Linderman RG (1986) Selective influence on populations of rhizosphere or rhizoplane bacteria and actinomycetes by mycorrhizas formed by *Glomus fasciculatum*. Soil Biol Biochem 18:191–196
72. Cameron DD, Neal AL, van Wees SC, Ton J (2013) Mycorrhiza-induced resistance: more than the sum of its parts? Trends Plant Sci 18:539–545
73. Koller R, Rodriguez A, Robin C, Scheu S, Bonkowski M (2013) Protozoa enhance foraging efficiency of arbuscular mycorrhizal fungi for mineral nitrogen from organic matter in soil to the benefit of host plants. New Phytol 199:203–211
74. Siddiky MRK, Schaller J, Caruso T, Rillig MC (2012) Arbuscular mycorrhizal fungi and collembola non-additively increase soil aggregation. Soil Biol Biochem 47:93–99
75. Egamberdieva D, Jabborova D, Wirth S (2013) Alleviation of salt stress in legumes by co-inoculation with pseudomonas and rhizobium. In: Aroca N (ed) Plant microbe symbiosis: fundamentals and advances. Springer, New Delhi, pp 291–303
76. Hashem A, Alqarawi AA, Radhakrishnan R, Al-Arjani ABF, Aldehaish HA et al (2018) Arbuscular mycorrhizal fungi regulate the oxidative system, hormones and ionic equilibrium to trigger salt stress tolerance in *Cucumis sativus* L. Saudi J Biol Sci 25:1102–1114

77. Hidri R, Mahmoud OMB, Debez A, Abdelly C, Barea JM et al (2019) Modulation of C: N: P stoichiometry is involved in the effectiveness of a PGPR and AM fungus in increasing salt stress tolerance of *Sulla carnosa* Tunisian provenances. Appl Soil Ecol 143:161–172
78. Igiehon NO, Babalola OO, Cheseto X, Torto B (2020) Effects of rhizobia and arbuscular mycorrhizal fungi on yield, size distribution and fatty acid of soybean seeds grown under drought stress. Microbiol Res 242:126640
79. Vosátka M, Látr A, Gianinazzi S, Albrechtová J (2012) Development of arbuscular mycorrhizal biotechnology and industry: current achievements and bottlenecks. Symbiosis 58:29–37
80. Chen M, Arato M, Borghi L, Nouri E, Reinhardt D (2018) Beneficial services of arbuscular mycorrhizal fungi–from ecology to application. Front Plant Sci 9:1270
81. http://www.teriin.org
82. https://www.mordorintelligence.com/industry-reports/mycorrhiza-based-biofertilizer-market
83. Adholeya A, Tiwari P, Singh R (2005) Large-scale inoculum production of arbuscular mycorrhizal fungi on root organs and inoculation strategies. In: Declerck S, Fortin JA, Strullu DG (eds) *In vitro* culture of mycorrhizas. Springer, Berlin, Heidelberg
84. Pal S, Singh HB, Farooqui A, Rakshit A (2016) Commercialization of arbuscular mycorrhizal technology in agriculture and forestry. Agriculturally Important Microorganisms:97–105
85. Verma A, Adholeya A (1996) Cost-economics of existing methodologies for inoculum production of vesicular-arbuscular mycorrhizal fungi. In: Mukerji KG (ed) Concepts in mycorrhizal research. Springer, Dordrecht, pp 179–194
86. Ijdo M, Cranenbrouck S, Declerck S (2011) Methods for large-scale production of AM fungi: past, present, and future. Mycorrhiza 21:1–16
87. Clement CR, Hopper MJ, Canaway RJ, Jones LHP (1974) A system for measuring the uptake of ions from flowing solutions of controlled composition. J Exp Bot 25:81–99
88. Peterson LA, Krueger AR (1988) An intermittent aeroponics system. Crop Sci 28:712–713
89. Mohammad A, Khan AG, Kuek C (2000) Improved aeroponic culture of inocula of arbuscular mycorrhizal fungi. Mycorrhiza 9:337–339
90. Schenck NC (1982) Methods and principles of mycorrhizal research. American Phytopathology Society, St. Paul. Minnesota, USA, pp 47–54
91. Abd Ellatif S, Ali EAM, Senousy HH, Razik ESA (2019) Production of arbuscular mycorrhizal fungi using in vitro root organ culture and phenolic compounds. J Pure Appl Microbiol 13: 1985–1994
92. Chandran PR, Potty VP (2009) Arbuscular mycorrhizal fungal inoculum production using Ipomoea batatas hairy roots in bioreactor. The Icfai University J Biotechnol 3:56–64
93. Hawkins HJ, George E (1997) Hydroponic culture of the mycorrhizal fungus *glomus mosseae* with *Linum usitatissimum* L., *Sorghum bicolor* L. and *Triticum aestivum* L. Plant Soil 196: 143–149
94. Lee YJ, George E (2005) Development of a nutrient film technique culture system for arbuscular mycorrhizal plants. Hort Sci 40:378–380
95. Tajini F, Suriyakup P, Vailhe H, Jansa J, Drevon JJ (2009) Assess suitability of hydroaeroponic culture to establish tripartite symbiosis between different AMF species, beans, and rhizobia. BMC Plant Biol 9:73
96. Elmes RP, Mosse B (1984) Vesicular-arbuscular endomycorrhizal inoculum production II experiments with maize (*Zea mays*) and other hosts in nutrient flow culture. Can J Bot 62: 1531–1536
97. Nuutila AM, Vestberg M, Kauppinen V (1995) Infection of hairy roots of strawberry (*Fragaria x Ananassa* Duch.) with arbuscular mycorrhizal fungus. Plant Cell Rep 14:505–509
98. Hung LLL, Sylvia DM (1988) Production of vesicular-arbuscular mycorrhizal fungus inoculum in aeroponic culture. Appl Environ Microbiol 54:353–357
99. Dugassa DG, Grunewaldt-Stöcker G, Schönbeck F (1995) Growth of *Glomus intraradices* and its effect on linseed (*Linum usitatissimum* L.) in hydroponic culture. Mycorrhiza 5:279–282
100. Jarstfer AG, Farmer-Koppenol SDM, Sylvia DM (1988) Tissue magnesium and calcium affect arbuscular mycorrhiza development and fungal reproduction. Mycorrhiza 7:237–342

101. Tiwari P, Adholeya A (2002) *In vitro* co-culture of two AMF isolates *Gigaspora margarita* and *Glomus intraradices* on Ri T-DNA transformed roots. FEMS Microbiol Lett 206:39–43
102. Bi Y, Li X, Wang H, Christie P (2004) Establishment of monoxenic culture between the arbuscular mycorrhizal fungus *Glomus sinuosum* and Ri T-DNA-transformed carrot roots. Plant Soil 261:239–244
103. Chandran RP, Potty VP (2010) In vitro co-culture of VAM fungi *Glomus microcarpum* in Ri T-DNA transformed hairy roots of *Vigna vexillata*. IJAEB 3:333–338
104. Pratap Chandran R, Potty VP (2011) Initiation of hairy roots from *Canavalia* sp. using *Agrobacterium rhizogenes* 15834 for the co-cultivation of Arbuscular mycorrhizal fungi, *Glomus microcarpum*. J Agric Technol 7:235–245
105. Nanak S, Virat AO, Ambani V (2017) An evaluation of large-scale production of AMF inoculum through root organ culture. Afr J Agric 4:278–286
106. Norris JR, Read DJ, Varma AK (1992) Methods in microbiology, vol 23. Academic press, UK
107. Gaur A, Adholeya A (2002) Arbuscular-mycorrhizal inoculation of five tropical fodder crops and inoculum production in marginal soil amended with organic matter. Biol Fertil Soils 35(3): 214–218
108. Gaur A, Adholeya A (2000) Effects of the particle size of soil-less substrates upon AM fungus inoculum production. Mycorrhiza 10:43–48
109. Millner PD, Kitt DG (1992) The Beltsville method for soilless production of vesicular-arbuscular mycorrhizal fungi. Mycorrhiza 2:9–15
110. Neumann E, George E (2005) Extraction of extraradical arbuscular mycorrhizal mycelium from compartments filled with soil and glass beads. Mycorrhiza 15:533–537
111. Douds DD Jr, Nagahashi G, Pfeffer PE, Reider C, Kayser WM (2006) On-farm production of AM fungus inoculum in mixtures of compost and vermiculite. Bioresour Technol 97:809–818
112. Plenchette C, Fortin JA, Furlan V (1983) Growth response of several plant species to mycorrhizae in a soil of moderate P-fertility. I. Mycorrhizal dependency under field conditions. Plant Soil 70:199–209
113. Feldmann F, Grotkass C (2002) Directed inoculum production–shall we be able to design populations of arbuscular mycorrhizal fungi to achieve predictable symbiotic effectiveness? In: Gianinazzi S, Schüepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture. Birkhäuser, Basel, pp 261–279
114. Mohammad A, Mitra B, Khan AG (2004) Effects of sheared-root inoculum of *glomus intraradices* on wheat grown at different phosphorus levels in the field. Agric Ecosyst Environ 103:245–249
115. Pringle A, Bever JD, Gardes M, Parrent JL, Rillig MC, Klironomos JN (2009) Mycorrhizal symbioses and plant invasions. Annu Rev Ecol Evol Syst 40:699–715
116. Bünemann EK, Schwenke GD, Van Zwieten L (2006) Impact of agricultural inputs on soil organisms—a review. Soil Res 44:379–406
117. Cipollini D, Rigsby CM, Barto EK (2012) Microbes as targets and mediators of allelopathy in plants. J Chem Ecol 38:714–727
118. Bender SF, Wagg C, van der Heijden MG (2016) An underground revolution: biodiversity and soil ecological engineering for agricultural sustainability. Trends Ecol Evol 31:440–452
119. Gao X, Guo H, Zhang Q, Guo H, Zhang L et al (2020) Arbuscular mycorrhizal fungi (AMF) enhanced the growth, yield, fiber quality and phosphorus regulation in upland cotton (*Gossypium hirsutum* L.). Sci Rep 10:1–12
120. Pellegrino E, Piazza G, Arduini I, Ercoli L (2020) Field inoculation of bread wheat with *Rhizophagus irregularis* under organic farming: variability in growth response and nutritional uptake of eleven old genotypes and a modern variety. Agronomy 10:333
121. Lehmann A, Veresoglou D, Leifheit EF, Rillig MC (2014) Arbuscular mycorrhizal influence on zinc nutrition in crop plants–a meta-analysis. Soil Biol Biochem 69:123–131
122. Bona E, Cantamessa S, Massa N, Manassero P, Marsano F et al (2017) Arbuscular mycorrhizal fungi and plant growth-promoting pseudomonads improve yield, quality and nutritional value of tomato: a field study. Mycorrhiza 27:1–11

123. Lumley S (2013) Sordid boon? The context of sustainability in historical and contemporary global economies. Academic Press
124. Nugent RA (2001) Using economic analysis to measure the sustainability of urban and peri-urban agriculture: A comparison of cost-benefit and contingent valuation analyses. In Workshop on Appropriate Methodologies in Urban Agriculture Research, Planning, Implementation and Evaluation (pp. 1–7)
125. Hijri M (2016) Analysis of a large dataset of mycorrhiza inoculation field trials on potato shows highly significant increases in yield. Mycorrhiza 26:209–214
126. Gupta MM, Abbott LK (2021) Exploring economic assessment of the arbuscular mycorrhizal symbiosis. Symbiosis 83:143–152
127. Gazey C, Abbott LK, Robson AD (2004) Indigenous and introduced arbuscular mycorrhizal fungi contribute to plant growth in two agricultural soils from South-Western Australia. Mycorrhiza 14:355–362
128. Ryan MH, Graham JH (2018) Little evidence that farmers should consider abundance or diversity of arbuscular mycorrhizal fungi when managing crops. New Phytol 220:1092–1107
129. Abbott LK & Lumley S (2014) Assessing economic benefits of arbuscular mycorrhizal fungi as a potential indicator of soil health. In Mycorrhizal fungi: Use in sustainable agriculture and land restoration (pp. 17–31). Springer, Berlin, Heidelberg
130. Chave M, Angeon V, Paut R, Collombet R, Tchamitchian M (2019) Codesigning biodiversity-based agrosystems promotes alternatives to mycorrhizal inoculants. Agron Sustain Dev 39:1–12
131. Rillig MC, Aguilar-Trigueros CA, Camenzind T, Cavagnaro TR, Degrune F et al (2019) Why farmers should manage the arbuscular mycorrhizal symbiosis. New Phytol 222:1171–1175
132. Pandit R, Parrotta JA, Chaudhary AK, Karlen DL, Vieira DLM et al (2020) A framework to evaluate land degradation and restoration responses for improved planning and decision-making. Ecosyst People 16:1–18
133. Boyle KJ (2017) Contingent valuation in practice. In: Champ PA, Boyle KJ, Brown TC (eds) A primer on nonmarket valuation. Springer, Dordrecht, pp 83–131
134. Kokkoris VA (2019) Establishment persistence and efficacy of commercial arbuscular mycorrhizal fungi (Doctoral dissertation, University of British Columbia)
135. Holland T, Vukicevich E, Thomsen C, Pogiatzis A, Hart M (2018) Arbuscular mycorrhizal fungi in viticulture: should we use biofertilizers? Catalyst: Discovery into Practice 2:59–63
136. Crossay T, Majorel C, Redecker D, Gensous S, Medevielle V et al (2019) Is a mixture of arbuscular mycorrhizal fungi better for plant growth than single-species inoculants? Mycorrhiza 29:325–339
137. Affokpon A, Coyne DL, Lawouin L, Tossou C, Agbèdè RD et al (2011) Effectiveness of native west African arbuscular mycorrhizal fungi in protecting vegetable crops against root-knot nematodes. Biol Fertil Soil 47:207–217
138. Schwartz MW, Hoeksema JD, Gehring CA, Johnson NC, Klironomos JN et al (2006) The promise and the potential consequences of the global transport of mycorrhizal fungal inoculum. Ecol Lett 9:501–515
139. Sosa-Hernández MA, Leifheit EF, Ingraffia R, Rillig MC (2019) Subsoil arbuscular mycorrhizal fungi for sustainability and climate-smart agriculture: a solution right under our feet? Front Microbiol 10:744
140. Powell JR, Rillig MC (2018) Biodiversity of arbuscular mycorrhizal fungi and ecosystem function. New Phytol 220:1059–1075
141. Krüger M, Stockinger H, Krüger C, Schüßler A (2009) DNA-based species level detection of Glomeromycota: one PCR primer set for all arbuscular mycorrhizal fungi. New Phytol 183: 212–223
142. Stockinger H, Krüger M, Schüßler A (2010) DNA barcoding of arbuscular mycorrhizal fungi. New Phytol 187:461–474
143. Angelard C, Tanner CJ, Fontanillas P, Niculita-Hirzel H, Masclaux F et al (2014) Rapid genotypic change and plasticity in arbuscular mycorrhizal fungi is caused by a host shift and enhanced by segregation. ISME J 8:284–294

Role of Ectomycorrhizal Fungi in Human Welfare

2

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Abstract

Anthropogenic and natural activities-based ecosystem disturbances lead to left-over degraded barren lands. Ectomycorrhizal (ECM) fungi play an important role in regulating the nutrient balance of the terrestrial ecosystem, assisting plant regeneration on disturbed lands by conferring improved tolerance against various biotic and abiotic stresses. There are several challenges and knowledge gaps in utilizing the ECM fungi for large-scale ecosystem restoration programs. For strengthening the successful utilization of ECM fungal applications in out-fields, this chapter has summarized the various applications, mechanisms, and considerable important parameters of ECM symbiosis in regenerating and improving host plant growth against diverse stress conditions such as drought stress, heavy metal stress, and forest fire, thus leading to the rehabilitation of degraded lands. Above all, the major challenge remains the production of large-scale ECM fungal inocula for restoration programs. This chapter has summarized the various ECM inoculation techniques, including their merits and demerits. This chapter also highlights the essential role of ECM fungi in nutrient dynamics of host plants with soil and ECM fungal application as biofertilizers in agriculture and commercial nurseries, thereby replacing harmful chemical fertilizers.

Keywords

Ectomycorrhizal fungi · Host plants · Rehabilitation programs · Stress tolerance · Nutrient cycling

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2.1 Introduction

Several anthropogenic activities result in degraded and barren lands [1]. Human advancements such as industrialization and advanced technologies increase heavy metal pollution in soil [2]. Apart from severely damaging plant growth, metal pollution also affects the immune system [3], human health [4], and soil microbiome [5] and also can be leached down to groundwater [6]. Anthropogenic activities also affect the earth's balance between incoming and outgoing solar radiations leading to an increase in temperature [7], which results in enhanced cases of drought [8]. The population increase and water scarcity emerge as new challenges to fulfill crop production and food safety [9]. Among natural disturbances of the ecosystem, a forest fire is one of the severe global disturbances [10, 11] which deleteriously affect soil microbial community and plant growth efficiency on burned lands [12, 13]. Several reports and evidence highlight ECM fungal potency as a strong biotechnological tool for bioremediation of human or naturally disturbed degraded lands.

Ectomycorrhizal (ECM) fungi are the ubiquitous plant symbionts of temperate and boreal biomes colonizing the roots of woody plants such as *Picea*, *Pinus*, *Acacia*, *Eucalyptus*, and *Larix* [14]. Most of the ECM fungi belongs to *Basidiomycota* and *Ascomycota* [15] and plays a very crucial role in nutrient cycling of the terrestrial ecosystem by exchanging soil nitrogen (N), phosphorus (P), potassium (K), and water with plants in return of their photosynthetically drawn carbohydrates [16]. ECM fungi are the main drivers of N and C dynamics in the forest ecosystem [17] and significantly influence the growth and survival of other soil microorganisms, including saprophytic fungi [18–20].

ECM fungal traits are crucial in afforestation programs for biodiversity conservation and forest management [21]. ECM fungi help to regenerate post-fire burned lands [22] and restore agricultural exploited lands [23]. They can also function as biofertilizers to replace the harmful chemical fertilizers in nurseries and agriculture [24]. ECM fungi play a critical role in establishing and enhancing trees' growth under a primary succession of sand dunes [25] and volcanic desert [26]. The efficient defense mechanisms of ECM fungi against metal stress [27–29] and their root colonization chemistry [30] enable their host plants to withstand the toxic metal concentrations and drought stress, respectively.

This chapter reviews and examines the ECM applications and roles in rehabilitating natural or human-mediated degraded lands. The chapter will give insight into (i) ECM role in nutrient cycling of terrestrial ecosystems, (ii) ECM symbiosis-based mechanisms behind improved efficiency of their host plants in tolerating various stress conditions, and (iii) various techniques to inoculate ECM fungi with plants roots under large-scale rehabilitation programs. An attempt has been made to look at the gaps, important aspects, and parameters required to make this symbiosis functionally applicable in out-fields, thus emerging as a more robust biotechnological tool.

2.2 Nutrient Cycling of Ectomycorrhizal Fungi

Autotrophs have restricted direct access to soil nutrients such as P and N [31]. In forest soils, N exists as part of different organic complexes, predominately proteins and amino acids [32]. The inorganic and organic phosphates are found as orthophosphate esters, inositol phosphates, and organic polyphosphates [34, 33]. ECM fungi play an essential role in nutrient dynamics of forest ecosystems by mobilizing unavailable soil nutrients to their host plants through nutrient mineralization in exchange for plant photosynthetic products [14, 35, 36]. The addition of nutrients in the soil alters the fungal behavior and shows plants to be more independent of fungi by not facilitating symbiotic association. The growth of ECM fungus associated with the *Pinus pinaster* plant is more likely to be independent of its fungal partner in case of applied fertilizers when nutrients are easily accessible, but the absence of fertilizers makes the plants grow prominently dependent on a fungal partner [37].

2.2.1 Carbon Cycling

One of the earliest attempts by Melin and Nilsson [38] showed that the delivery of labeled $^{14}CO_2$ to the leaves of plants associated with ECM fungi results in ^{14}C appearance in the fungal mantle within the next day. In symbiosis, ECM fungi increase plant carbohydrate attraction toward root-fungal mycorrhizae and plant roots by the factor of 4–9 and 15–19, respectively [39, 40]. Host plant transfers the carbon (C) to their fungal partner in the form of simple soluble sugars, amino acids, and carboxylic acids [41]. The host roots secrete sucrose in fungal-plant common apoplast, which gets hydrolyzed by the plant-derived enzyme invertase bound to their cell wall [42–44]. The hydrolysis of apoptotic sucrose by invertase forms glucose and fructose in a 1:1 ratio followed by fungal preferential uptake of glucose over fructose, ultimately leading to fructose abundance in the common apoplast [43, 45]. The accumulated fructose in the common apoplast regulates invertase activity, as this gets inhibited in the host by fructose [43]. The surrounding sugar concentration is crucial in controlling some ECM fungal genes upon symbiosis. The ECM fungus *Amanita muscaria* upregulated the monosaccharide transporter gene (*AmMst1*) in symbiotic association with its host, enhancing sugar uptake [45].

Interestingly, in *Laccaria bicolor*, out of 15 predicted hexose transporters, six genes were found to be strongly upregulated in symbiosis, and only three out of six were proved to be hexose transporters in symbiosis [46]. The majority of the above genes showed independent sugar regulation in symbiosis, dividing them into two gene sets. One set is associated with the carbohydrate uptake by soil growing hyphae to use soil as an additional carbon source and prevent sugar leakage in starving conditions. Another group is associated with carbon uptake in fungal-plant symbiosis. Further, for the maintenance of carbohydrate sink in fungal partner, ECM fungi immediately utilize the obtained carbon in adenosine triphosphate (ATP) production, in amino acid biosynthesis, and for generating storage compounds such as glycogen

(nonmobile, long-term storage), trehalose, and mannitol (mobile, short-term storage). Hence, the sugar export in symbiosis is regulated by controlling (i) sugar efflux at the common apoplast, (ii) hydrolysis of sucrose by invertase, and (iii) root and fungal competition for hexoses in the common apoplast [47]. Interestingly, the host plant carbon allocation to the ECM fungi is not dependent on the amount of N the plant receives from ECM fungi [48–50]. Both high and low N transferring ECM fungal species receive similar C content from their host plants [49]. The identification of possible factors behind this exchange needs further investigation.

2.2.2 Nitrogen Cycling

Though N is abundantly present in organic forms (amines, amides, heterocyclic N compounds, lipids, polysaccharides, and other decomposition products in soil organic matter) as well as mineral forms (NH_4^+ and NO_3^-), there is limited access for forest trees [32, 51–53]. Besides the limited decomposition capabilities of ECM fungi, some studies have shown that ECM fungi retrieve N from inorganic sources and facilitate the plant access to organic forms of nitrogen present in soil organic matter (SOM) under N-depleted conditions [54]. The addition of excess inorganic N in the soil surrounding the host plant disturbs their mycorrhizal symbiosis and decreases the ECM fungal diversity [55]. The accumulation of SOM increases with the forest age, and the source of N also gets dominated in organic forms by this succession of the forest [56]. The ECM fungi secrete various extracellular proteases to degrade proteins for N mobilization. The aspartic peptidases are the most dominant enzymes secreted by different ECM fungi such as *Hebeloma crustuliniforme* [57], *A. muscaria* [58], *L. bicolor* [59], *Paxillus involutus* [60], and *Suillus* strains [61]. The protease activity of ECM fungi plays a role in N assimilation which is positively correlated with the availability of N sources, where carbon source availability also acts as an additional regulator of fungal protease activity. A large proportion of organic N in soil is associated with minerals [62]. Further, the protein compounds of SOM show firm binding to the mineral surface [63]. The SOM association with minerals makes SOM stabilized and recalcitrant to the enzymatic decomposition by soil microorganisms [64, 65]. Recent studies suggested that N assimilation from protein complexes associated with minerals gets absorbed on the mineral surface, which is further facilitated by low molecular weight (LMW) organic complexes released by ECM fungi [62, 66]. The LMW complexes (metabolites) being reactive to the mineral surface prevent the protease binding to the mineral surface vacant sites and hence increase the probability of enzyme binding to target proteins only [66]. However, some studies showed oxalic acid as the potential candidate for the liberation of organic complexes from minerals [67, 64], but the exact nature of these LMW metabolites is not yet adequately studied. Besides that, the *Basidiomycetes* and *Ascomycetes*, the prime candidates of ECM fungi [15], have excellent capabilities to produce secondary metabolites [68]. Furthermore, the proteolytic hydrolysis of proteins associated with

minerals is independent of the LMW metabolite-based desorption step and is direct in many cases [66, 69].

2.2.3 Phosphorus Cycling

In forests, the P cycling is mainly regulated by soil microbial biomass instead of its annual uptake by trees [70, 71]. ECM fungi mobilize the recalcitrant P from the soil to plants by secreting organic acids (citrate, oxalate, and malate), phytases, phosphomonoesterases, and phosphodiesterases [72, 73]. The P mineralization also involves the cleavage of ester bonds by fungal extracellular phosphatases to process unavailable phosphate for plant intake [74, 75]. ECM symbiotic trees are more efficient in utilizing nutrients in organic forms than the trees associated with arbuscular mycorrhizal (AM) fungi [76]. The dominant organic forms of P in soil are mainly found as phosphate monoesters, phosphate diesters, and inositol phosphate [77], and phosphatase activity has also been reported from ECM root tips frequently [78, 79]. In ECM plots, the community structure of soil fungi mediates the high enzymatic activities of fungi which leads to the occurrence of higher organic P complexes in ECM plots compared to AM fungal plots. The difference in fungal community structure on ECM and AM plots results in their varied phosphatase activity and availability of organic and inorganic forms of P [70]. Even though saprotrophic and ECM dominated assemblages of forest ecosystem showed similar oxidase and phosphatase activities, there is a slight difference in peroxidase and phosphodiesterase activities, thus suggesting the ECM fungal roles in increasing soil P availability for their host plants [80]. ECM fungi can also uptake the soil inorganic P through their P transporters, where the transporter activities vary depending upon different types of P transporters and P availability [81, 82]. Numerous inorganic P transporters have been reported from ECM fungi [83, 84]. The phosphatase activity of ECM fungi catalyzes the inorganic P release out from organic P complexes present in the organic matter [85], where the fungal phosphatase activity got enhanced under the P-deficient conditions in their host plants [86].

2.2.4 ECM Fungal Decomposition and Turnover

The decomposition of ECM fine roots can deliver a massive amount of nutrients to the soil [87] and significantly influence the C and N cycling in an ecosystem [87, 88]. The decomposition of ECM fungi can be carried out by (i) autolysis by ECM fungus itself; (ii) bacteria typically from genera *Pseudomonas*, *Duganella*, *Ewingella*, *Pedobacter*, *Stenotrophomonas*, and *Variovorax*; and (iii) fungal genera such as *Penicillium*, *Cladosporium*, *Mortierella*, and *Aspergillus* and yeast [89, 90]. Microarthropods such as *Folsomia candida* can further facilitate this, which graze upon ECM fungi [92, 91]. The decomposition rates of ECM fungi are determined by several factors such as diversity of ECM community, cell wall composition, morphology, N concentration, and soil temperature. Melanin is present

in fungal cell walls as a complex polymer [93]; its concentration varies in different species of ECM fungi. The decomposition rates of ECM fungi are higher with a high concentration of N, chitin, and lower amounts of melanin [94]. The N richness in ECM fungi is a preferred target for decomposers in N-limited forest soils [95]. Soil temperature determined by trees canopy and climatic changes is positively correlated with the decomposition of ECM fungi [87, 96]. The ECM fungi with distinct morphology have different decomposition rates and are also significantly influenced by the composition of the ECM community [87].

2.3 Biofertilizers

In sustainable farming, biofertilizers, an addition to chemical fertilizers, increase plant production and fertility of the soil [24]. For decades, mycorrhizal plants have been well reported for their improved uptake of soil nutrients and water compared to non-mycorrhizal plants [31]. The symbiotically associated ECM fungus, *Hebeloma hiemale*, has a more positive impact on *Prunus* plant growth parameters than the control, compost, and chemical fertilizers under greenhouse conditions [24]. ECM fungi can also act as a bio-fungicide mainly based on their property to produce an antifungal chitinase enzyme. Among different ECM fungal species tested, *Suillus* species possess a high chitinase activity, whereas the indole-3-acetic acid (IAA) production is highest in *Laccaria* species. The IAA production from ECM fungi causes enhanced growth of their host plant seedlings in nurseries and hence highlights them as biofertilizers [97]. The ECM inoculation of *Eucalyptus* clone cuttings in commercial nurseries reduces the dose of P fertilizer required for host growth [98]. Trees growing in poor phosphorus soils show stomatal conductance and substomatal CO_2 content similar to drought stress-induced responses in plants [99, 100]. Thus, P-deficient trees are more vulnerable to drought and other abiotic stresses than P-rich trees [100]. The ECM fungal inoculation with host plants efficiently enhances the plant P uptake compared to non-mycorrhizal plants, as shown in Table 2.1, thus promoting plant growth. The transformation of symbiotically associated ECM fungi from supplier to the consumer of nutrients in unfavorable conditions might be the reason for the decreased P leaf content upon endo- and ECM fungal symbiosis [101].

Nitrogen has a crucial role in the plant photosynthetic system. Adequate availability of N enhances the photosynthesis efficiency and thus ameliorates the water use efficiency calculated as ratio of photosynthesis rate to transpiration rate [109]. Inoculation of *Pisolithus tinctorius* improves the physiological state of the host plant *Quercus suber* by increasing its N uptake, leading to increased photosynthesis and water use efficiency of plants [108]. The potential of ECM fungal inocula in increasing N content of *Pinus pinaster* needles significantly varied with the fungal species, individual or co-inoculation of multiple ECM species, and phenotypic superiority of host plants [107]. The potassium (K) transfer efficiency of ECM fungi from soil to host plant depends upon ECM species and the available external concentration of K [104]. They also indicate the host plant species and its growth

Table 2.1 Improvement in nutrient uptake by host plants due to mycorrhizal symbiosis by different ectomycorrhizal fungi

ECM fungi	Host	Inoculation method	Effects	References
Inocybe rimosa, *Amanita crocea* Singer, *Tricholoma* sp., *Boletus comptus* Simonin	*Quercus brantii*	Hydroponic culture system	Increase in P uptake of host plants	[102]
Pisolithus sp., *Rhizopogon* sp., *Scleroderma* sp.	*Prunus avium*	Thin, concentrated, suspended powder of mycorrhizal propagules as inoculum	Increased potassium (K) uptake and decreased P content in leaf	[101]
Suillus indicus *Suillus sibiricus* assisted with biochar prepared from pine needles	*Pinus wallichiana*	Mycelial suspension in sterile water	Enhanced ECM colonization, nutrient uptake, and growth of host seedling	[103]
Hebeloma cylindrosporum *Paxillus ammoniavirescens* *Laccaria bicolor*	*Pinus taeda* *Pinus taeda* *Pinus taeda*	Homogenized mycelia suspended in a liquid	Increased shoot K irrespective of external availability Increased shoot K irrespective of external availability Increased shoot K in sufficient external K	[104]
Lactarius sp., *Scleroderma* sp., *Lycoperdon* sp.	*Pinus elliottii*	Fungal liquid culture	Increased plant growth and absorption of N, P, and K in plant	[105]
Amanita sp., *Scleroderma* sp., *Elaphomyces* sp.	*Eucalyptus urophylla*	Homogenized fungal mycelium inserted in calcium alginate beads	Increased plant growth, ECM colonization, plant N and K uptake	[106]
Suillus bovines + *Laccaria laccata* + *Lactarius deterrimus*	*Pinus pinaster*	Mycelial or spore suspension	Increased plant biomass and N content of pine needles	[107]
Pisolithus tinctorius	*Quercus suber*	Peat-vermiculite growth substrate colonized with fungal mycelium	Increased N uptake and photosynthesis in host plants	[108]

conditions as the critical determining factors for their benefits from given ECM fungus. The combined application of ECM inoculation and exogenous calcium (Ca) on plant seedlings resulted in magnified mycorrhizal benefits to the host plant

[110]. Based on the minor difference in ECM fungal richness reported from secondary forest restored from agricultural land and primary forests dominated with Korean pine, the restoration of agricultural land with nursery-grown plant seedling showed the great potential of ECM fungi to re-establish themselves on prolonged agricultural exploited lands in two decades [23]. The efficiency of ECM fungal inocula in improving the nutrient status of host plants under different stress conditions has also been discussed in the subsequent sections.

2.3.1 Effect of Different Fertilizers on Natural ECM Fungal Community

Several environmentally safe fertilizers have been introduced as alternatives to chemical fertilizers, including organic fertilizers, mineral fertilizers, and effective microorganisms (EM) mix. The utilization of EM mix to promote plant growth negatively impacts ECM fungal diversity and abundance, whereas the organic and mineral fertilizers remain safe for ECM diversity [111]. ECM fungi have an essential ecological role in the terrestrial ecosystem, and the different fertilizers significantly affect the soil ECM community. Here we have collected the various reports regarding the effects of soil fertilization and fertilization period on soil natural ECM fungal community (Table 2.2).

The ECM fungal richness is correlated with the density of plant fine roots, and no change in roots in K fertilization highlights later null effect on ECM fungal richness [112]. Wood ash fertilizer, a rich source of potassium [119], alters the plant root growth pattern and soil chemistry, thus changing the richness of the ECM fungal community [117]. In response to P and N limitation, symbiotic plants enhanced the C allocation to their ECM fungal partners to maintain K assimilation from soil [120]. The higher N input in soil due to N deposition or N fertilizers causes the elevation in N leaching [116, 121] and reduces the forest retention capacity [122]. The reduction in ECM fungal production upon increased N content from atmospheric deposition throughout two consequent years [116] also supports the negative impact of N deposition on ECM production. The ECM fungal reduction could be the reason for the reduced C allocation to ECM fungi by plants sufficiently applied with N and P. The N leaching increases due to N fertilization. No increase in N leaching was found further by adding both N and P fertilizers. The study suggests that N retention capacity alleviates due to reduced ECM fungus while keeping ECM fungal retention capacity constant [116]. The P and N fertilizers enhance the ECM fungal relative abundance without affecting their richness. The combined N and P fertilizer causes altered relative abundance of different ECM fungi at the order level [115]. The N fertilization reduces the plant root P content regardless of soil P content due to declined plant C allocation to root-associated ECM fungi [123]. This reduces P uptake by roots and restricts P in fungal biomass only and thus negatively impacts the host plant nutrition. The N fertilizer could be accompanied by P fertilization to compensate for the N input-induced tree P deficiency [115].

Table 2.2 Studies showing effects of different fertilizers on soil natural ECM fungal community structure

Fertilizer	Treatment period	Host	Ecosystem	Effects on ECM diversity and abundance	References
Potassium fertilizer	~8 years	*Picea abies*	Forest	None	[112]
Nitrogen-, carbon-, and water-sufficient fertilized soil	Regular practice at nursery	Scots pine	Bare-root forest nursery	ECM community dominated by *Ascomycota* at 1-year-old seedlings	[113]
Compost as organic fertilizer	Unspecified	*Picea abies*	Forest nursery	Increases *Ascomycota* abundance in fine root colonization	[114]
Phosphorus fertilizer	~2.5 years	*Fagus sylvatica*	Forest	Relative abundance increased with no effect on fungal richness	[115]
Nitrogen fertilizer	~2.5 years	*Fagus sylvatica*	Forest	Relative abundance increased with no effect on fungal richness	[115]
Nitrogen fertilizer	4 months	Norway spruce forest (32 years old)	Forest	Reduced ECM production	[116]
Nitrogen fertilizer + phosphorus fertilizer	4 months	Norway spruce forest (32 years old)	Forest	Reduced ECM production	[116]
Woo ash fertilizer	12 years	Conifer forest stands	Forest	Increased and altered ECM fungal diversity	[117]
Effective microorganisms mix of naturally occurring and beneficial microorganism	~4 months	*Fagus sylvatica Quercus robur*	Bare-root forest nursery	Negatively effected	[111]
Organic fertilizer (Actifos)	~4 months	*Fagus sylvatica Quercus robur*	Bare-root forest nursery	Neutral	[111]

(continued)

Table 2.2 (continued)

Fertilizer	Treatment period	Host	Ecosystem	Effects on ECM diversity and abundance	References
Mineral fertilizer (Busz Forte)	~4 months	*Fagus sylvatica Quercus robur*	Bare-root forest nursery	Neutral	[111]
Forest litter	2 years	Pine seedlings	Nursery	Increases suilloid mycorrhiza abundance	[118]

Interestingly, the ECM community can respond to nutrition imbalances by altering the relative abundance of different ECM fungi at the order level. For example, their P sequestration ability increased the relative abundance of root-associated fungi [124]. The ECM fungal community developed on young plant seedlings in nurseries is characterized by the dominance of *Ascomycota* over the *Basidiomycota* [113, 125, 126]. The Scots pine seedlings at barefoot forest nurseries dominated by *Ascomycota* ECM fungi on 1-year-old seedlings got replaced by the *Basidiomycota* in successive years. A high occurrence of *Ascomycota* on young seedlings is correlated with the high fertilization and water availability of nursery soil, as shown in Table 2.2, which also suggests the varying life strategies of ECM fungi [113]. The pre-treatment of pine litter with plant seedlings results in better ECM fungal community structure, as shown in Table 2.2, and root development. Later, it results in more occupied soil volume and thus enhanced water and nutrient uptake, ultimately increasing their survival rates upon out-plantations [118].

The inoculation with a single ECM fungus and multiple combinations gives varied plant growth results and nutrient uptake potential. The studies emphasized co-inoculation of numerous ECM fungi in specific combinations for better results compared to inoculation with a single candidate only [127–129]. The colonization of ECM fungi with some host plants can be improved by co-plantation of ECM fungal-specific host plants, suggesting the selection of compatible ECM fungi for restoration programs [130]. It is common practice to inoculate plant seedlings with ECM fungi at nurseries for healthy symbiosis establishment before their out-plantations in the field. The major challenge is establishing symbiosis with specific desired species to prevent contamination with unwanted species [131]. The success of ECM inoculation depends on several factors, such as inoculum type, the viability of inoculants during storage, and infective potential [132].

2.4 Drought Stress Tolerance

Anthropogenic activities result in global warming with increased temperature [7], leading to increased drought cases [8]. It affects plant growth, seed germination, stomatal conductance, transpiration rate, and net photosynthetic rate [133, 134]. The large surface area and extended growth of fungal extramatrical hyphae for better water uptake are the direct mechanisms behind the improved growth of their host plants under a water-deficient environment [14, 135]. The longer the extramatrical hyphae, the higher the survival rates of host plants have been reported [136]. The ECM fungal partner has reported correlations with plant root hormones (abscisic acid and cytokinins) and aquaporins under drought stress in symbiosis. The cytokinin is correlated to the plant aquaporins, whereas abscisic acid to the fungal aquaporins, which possesses high water transportation potential [137]. On the other hand, earlier studies suggested abscisic acid as a crucial regulator of plant aquaporins [138, 139]. In water-deficient conditions, the water retained in tiny pores of soil becomes unreachable for plant roots where the symbiotically associated ECM fungal hyphae can grow and improve water uptake efficiency of host roots for their better survival [140].

Ca^{2+} is reported as a plant key signaling molecule under environmental stress. The Ca^{2+} signaling protects the plants from drought stress by regulating stomatal closure, root water absorptivity, and stress-responsive genes [141–143]. The drought conditions cause a significant reduction in the transfer of extractable Ca from soil to host plant, which is efficiently improved by establishing ECM fungal symbiosis with host plants and promoting Ca-based drought tolerance in host plants. The drought stress-induced malondialdehyde (MDA) content in the plants causes damage to their cell membrane. The synergism of *Suillus luteus* with *Trichoderma virens* in the inoculum results in reduced MDA levels in the host plant upon symbiosis. In addition to this, *T. virens* induces 1,3-glucanase and chitinase production in *S. luteus*, suggesting the former as a stimulating factor [144]. The nonstructural carbohydrate (NSC) content, total starch, and soluble sugar content tend to decrease in plants under drought stress which can be alleviated by establishing symbiosis of plants with ECM fungi. The ECM fungal symbiosis can protect plants from carbon starvation and water deficiency by increasing stomatal conductance, enhancing photosynthesis, and increasing tissue soluble sugar levels, resulting in enhanced water absorptivity [145]. The increase in assimilative nutrient enzymes by ECM symbiosis with plant seedlings results in improved plant growth under water stress with enhanced C regulation and better uptake of N and P [146]. Hence, the reports shown in Table 2.3 point toward the ECM fungi as a potential biotechnological tool to restore or enhance plant growth on drought-affected lands.

Table 2.3 Studies showing different mechanisms behind ECM symbiosis-based enhanced host plant survival under drought stress

ECM fungi	Assisting agent	Host plant	Mechanism	References
Suillus luteus	Calcium (Ca) fertilizers/ exogenous Ca^{2+}	*Pinus sylvestris* var. *mongolica*	Upregulation in antioxidant enzyme activities of host plants against drought induce reactive oxygen species	[147]
Xerocomus chrysenteron	Extractable Ca from soil water	*Quercus acutissima*	Enhance Ca transfer from soil water to plants	[148]
Suillus luteus	*Trichoderma virens*	*Pinus sylvestris* var. *mongolica* *Picea koraiensis*	Reduction in malondialdehyde content	[144]
Suillus variegates	None	*Pinus tabulaeformis*	Alleviating plant carbon starvation under severe drought stress	[145]
Pisolithus tinctorius	None	*Quercus suber*	Altered lipid composition of host chloroplast to maintain its integrity under drought stress	[149]
Pisolithus tinctorius	None	*Nothofagus dombeyi*	Enhanced plant growth and N and P content in ECM plants under water stress	[146]
Pisolithus tinctorius	None	*Nothofagus dombeyi*	Enhanced carbon production in ECM plant roots under drought stress	[150]

2.5 Heavy Metal Stress Tolerance

The heavy metal pollution in the soil is one of the significant environmental threats due to its accumulative nature and non-biodegradability. Both natural such as geologic parent material, volcanic eruptions, and wind-blown dust in deserts and anthropogenic activities such as agricultural, mining, sewage water, industrial, and transportation sources can be the reason for heavy metal pollution in the environment [151, 152]. From the total contents of heavy metals in the soil, 95% of arsenic (As) has been reported due to wastewater irrigation. In contrast, the large fractions of nickel (60%), manganese (88%), chromium (75%), and vanadium (76%) originate from natural sources. The significant proportions of copper (Cu) (81%), zinc (Zn) (70%), and lead (Pb) (93%) were reported to be accumulated from industrial origins [153]. The continuous intake of metal-polluted crops may cause severe effects in humans such as inhibition of ATP production and protein coagulation by As; kidney, liver, and brain malfunctioning by Pb; capillary damage and corrosion by Cu; and bloody urine, diarrhea, and vomiting by Zn [154–156].

ECM fungi can improve the survival of their host plants on metal-contaminated lands by minimizing the metal transfer to them [157]. In ultramafic soils of New

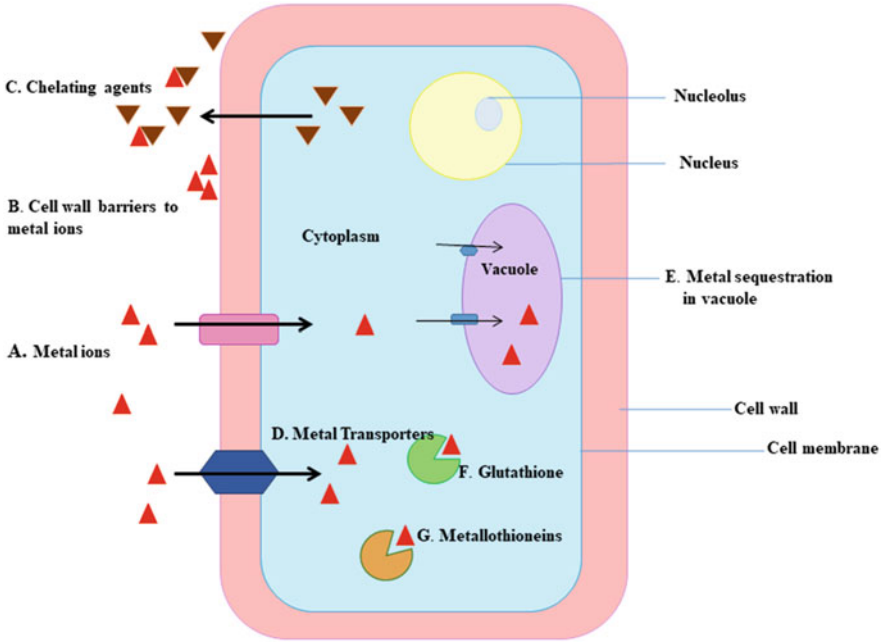

Fig. 2.1 Defense mechanisms of ECM fungi against metal stress. (**a**) Toxic high concentration of metal in the fungal exterior. (**b**) Cell wall barriers to prevent metal influx into cells. (**c**) Release of metal-chelating exudates from ECM fungi. (**d**) Metal influx through membrane transporters. (**e**) Metal sequestration in vacuole. (**f**) and (**g**) metal sequestration in the cytoplasm by glutathione and metallothioneins

Caledonia, the dominant symbiosis of ECM with plants is shown to be the primary reason for plant-enhanced tolerance against extreme soil conditions [29]. Inoculation of *Pisolithus albus* isolated from the ultramafic soils of New Caledonia from *Eucalyptus globulus* and *Acacia spirorbis* resulted in increased transportation of deficient essential elements from soil to host plants and further protected the plant from harmful effects of heavy metals. It has also been reported that ultramafic soils do not minimize but promote ECM diversity [158, 159]. The fungal cellular responses against metals are very diverse (Fig. 2.1) due to their different metal and species specificity, where huge differences were even observed within species [160, 161].

The mechanisms include the extracellular release of metal-chelating exudates such as oxalic acid, succinic acid, malic acid, and formic acid [27, 162]. The metal binding to cell wall components such as chitin, melanin, and cellulose prevents the metal influx [163, 164]. Further, the ECM fungi have efficient intracellular defense mechanisms such as membrane transporters for metal efflux and intracellular metal sequestering agents like metallothioneins (MTs), glutathione, and phytochelatins. The membrane ABC transporters, P-type ATPase, and a primary facilitator superfamily permease responsible for metal efflux are a strong defense against metal

toxicity in *P. albus* [165]. Metallothionein protein was first discovered by Margoshes and Vallee in 1957 [166] from the horse kidney cortex and had cadmium binding efficiency, which results in the accumulation of cadmium in the tissue [166]. MTs are the family of low molecular weight cysteine-rich conserved proteins, which bind to metal ions through the thiol group of their cysteine residues, playing a prime role in metal sequestration [167–169]. Numerous MTs have been reported for ECM fungi such as *LbMT1* and *LbMT2* from *Laccaria bicolor* [170], *SIMTa* and *SIMTb* from *Suillus luteus* [171], *PaMT1* from *P. albus* [172], and *AsMT1* from *Amanita strobiliformis* [173], which play essential roles in response to metal stress. Glutathione, an intracellular peptide, has high metal-binding efficiency with their thiol groups. It acts as a potential substance for oxidative stress prevention and its role as a precursor for phytochelatin biosynthesis [28, 174]. Phytochelatin from the peptide family also functions against heavy metal stress conditions [175]. The symbiosis of ECM fungi protects their host plants from heavy metal toxicity through their robust metal defense mechanisms. The ECM plants can accumulate more metal concentrations than non-ECM plants [176, 177]. Hachani et al. reported the reduced bioaccumulation of heavy metals in *Pinus halepensis* upon ECM symbiosis, thus enhancing phytostabilization efficiency [178]. Several studies also reported heavy metal accumulation in plants upon their symbiosis with ECM fungi, indicating ECM plants ameliorate phytoextraction-based phytoremediation of heavy contaminated lands [179, 180]. The differences in outcomes could be based on several factors such as heavy metal type, concentration in the outer environment, time of exposure, media used, and mycorrhization rates [178, 179].

Both edaphic properties and vegetation cover have shown a strong relation to fungal richness. ECM fungi were found relatively higher in woody plant-dominated lands than AM fungi. The higher fungal richness was found at mined land relative to non-mined land, which is possibly the outcome of stockpiling topsoil before mining forming composite soil mixture from different sites carrying high fungal richness [181]. AM fungi play essential roles in the early stages of mining rehabilitation through their associations with naturally restored *Acacia* species or grass. In contrast, the ECM associations come into dominance at later stages over the establishment of woody vegetation [182]. In mining site rehabilitation, *Scleroderma* sp., *Laccaria* sp., and *Pisolithus* sp. appear at initial stages, *Cortinarius* sp. at an intermediate stage, and *Russula* sp. at a later age. The *Russula* sp. prominently establishes symbiosis with mature trees and forests, which could be why they are late-stage ECM fungi. ECM fungal symbiosis is strongly related to aboveground vegetation [181]. In Lower Lusatia (Germany), the rehabilitation of lignite mining site with red oak attained dominant symbiosis of ECM fungus *Cenococcum geophilum* in almost all stands of the studied chronosequence [183].

2.6 Restoring Burned Lands

Forest fires are the natural and non-prescribed burning or combustion of plants leading to disturbance in the natural soil fungal community [184, 185]. The land burning causes removal of the organic soil layer and deposition of ashes leading to nutrient unavailability and creates partial soil sterilization, ultimately leading to the extinction of ECM fungi in local burned areas [186, 187]. Due to fire, the death or injury of ECM host plants causes an impact on ECM fungal communities [188]. Forest fire causes a significant reduction in host plants, ultimately leading to reduced host roots, habitat for ECM fungal colonization, and young open canopy [12], where ECM fungal diversity increases with the canopy closure [56]. Intense wildfires can damage ECM fungal community, root tips, and soil biota higher than prescribed burns [13]. Several species of ECM fungi have fire-resistant properties based upon fire frequency and dependency [189]. ECM fungi produce extracellular enzymes that play essential roles in nutrient cycling, such as peroxidases and laccases produced by some ECM fungi function in accessing the unavailable nutrients in dead wood debris [190]. Intense wildfires can create empty soil niches devoid of roots and ECM community, occupied by surrounding surviving fungi, causing an altered ECM fungal community. Fire-induced competition relief among ECM species can recruit ECM fungal species with different enzymatic properties and induce ECM fungi to produce an altered subset of enzymes based upon changed edaphic features post-fire [191, 192]. Post-fire, the subgroup of heat-resistant ECM fungal spores or sclerotia can survive on burned lands [193]. The colonization of new fungal species occurred due to the fire-induced release of fire competition [194]. Furthermore, the soil of burned land has lesser pH levels, high extractable P, less total N, but more mineral N (easily accessible by plants) than unburned. The fresh weight of plant seedlings grown in burned soil is half of the seedlings grown in unburned soil, which could be explained most likely by changed soil chemical and physical characteristics after burning [37].

The ECM fungi significantly differ in improving their host plant growth on unburned and burned soils. The efficiency of ECM fungal inoculum in increasing plant growth varies on burned and unburned soils. The increased P concentration in ECM-inoculated seedlings on burned soils compared to control could be the reason for enhanced *Pinus pinaster* growth in burned soils when associated with ECM fungi, *Rhizopogon roseolus*, *Suillus bovines*, and *Pisolithus tinctorius*. The negative correlation of ECM colonization and N concentration in the shoot [22, 195] supports the dependency of mycelial development on N, which ultimately causes the N dilution in plants [22]. The dominant ECM species of soil showed the innate potential for resilience in the post-fire lands, which is why many studies reported the same dominant ECM fungal species on both burned and non-burned land (Table 2.4) [196, 197]. It could take a long time for burned land to be colonized by the ECM fungal community present on prior fire land and even not observed after 16 years post-fire [12]. The burned lands are dominated by ascomycetes in early fungal communities, slowly replaced by basidiomycetes [198]. Post-fire, different ECM fungi were identified at various stages of the ECM fungal succession on

Table 2.4 Studies showing ECM fungal species different responses to post-fire conditions

ECM fungi	Description	Ecosystem	References
Rhizopogon olivaceotinctus	Abundance increases post-fire	*Pinus ponderosa* plots	[189]
Rhizopogon roseolus, *Cenococcum geophilum*	High frequency on both burned and unburned land	*Pinus sylvestris* forest	[196]
Wilcoxina rehmii	Dominant on both burned and unburned land	*Pinus ponderosa*	[197]
Hebeloma mesophaeum	Late colonizer of burned land (5–10 years)	*Pinus sylvestris* forest	[196]
Cortinarius sp.	Post-fire, abundance positively correlated to passing the time and reaches a maximum at 152 years' site	Boreal forest	[198]
Piloderma species	Post-fire, abundance highest at 42 years and decreases afterward	Boreal forest	[198]
Suillus species	Dominant species post-fire	*Larix gmelinii* forest	[12]

burned lands, as shown in Table 2.2. For example, *Rhizopogon olivaceotinctus* has been reported to increase after the fire [189]. At the nursery, the ECM inoculation with *Pinus pinaster* plants causes the enhanced growth of host plants compared to non-inoculated plants on burned plots. The selection of compatible ECM fungi to enhance the growth of host plants under stress conditions is an initial and essential step to consider carefully [199].

Several studies have been reported to identify fire-resistant ECM fungi that survived after the fire and to understand burned land chronosequence of the ECM fungal community. Besides the few studies regarding ECM fungal efficiency to enhance plant growth on burned land are available, extensive studies are lacking to date and hence are required to utilize the ECM fungi as a vital biotechnology tool for rehabilitation of burned lands.

2.7 Inoculation Technologies

The technology used to inoculate the ECM fungi with plant seedlings at commercial nurseries significantly impacts fungal efficiency to promote plant overall growth [132]. Broadly, the ECM fungal inocula used at commercial nurseries are of three different types: (i) natural inoculum such as soil and humus, (ii) ECM fungal spores, and (iii) vegetative mycelium-based inoculum [200].

Soil collected from tree plantations in natural forests containing the ECM propagules and roots were used as inocula in nurseries widely all around the globe [37, 201]. Inoculation of *Pinus tabulaeformis* seedlings with ECM-enriched

plantation soil results in enhanced plant growth and ECM colonization compared to non-soil treatment of seedlings. Soil inoculum could be used on a small scale only but is inconvenient for large-scale afforestation programs [202]. The risk of root-colonizing pathogens in soil inoculum is its another demerit [203]. The in vitro preparation of ECM fungal inoculums includes various techniques and methodologies [204]. The in vitro ECM inocula perform better than plantation soil inocula [205]. The crude inocula are prepared by mixing disinfected fine fragments of roots with their growing substrate such as soil followed by inoculation with fungal suspension [206]. Besides the higher efficacy and viability of fungal spores [207], the spore-based inoculation method has some demerits such as limited production of basidiospores based on its seasonality, taking a relatively long time to grow, and less spore viability [132, 208]. The optimal fungal isolate selection is not possible based upon spore inoculation [209], suggesting vegetative mycelium-based inoculation is better [132]. Also, the root colonization obtained by spore inocula is less than colonization obtained by ECM vegetative inoculation [210]. The vegetation mycelial inoculum, being able to colonize seedlings with specified ECM species, requires isolations and maintenance of pure cultures, selection of compatible ECM species, fungal inoculum preparation, and inoculation with seedlings [200]. The ECM mycelium slurry prepared by blending mycelia in a liquid medium is one of the primary ECM inocula used to inoculate plant seedlings and is applicable only for small-scale inoculum production [211, 212].

The large-scale production of ECM fungal inoculum remains the big challenge in its application-based utilization at commercial levels. The different techniques include (i) growing vegetative mycelium in peat-vermiculite mix [213], (ii) solid substrate fermentation-based ECM fungal propagation on growth medium [214], and (iii) submerged cultivation of ECM fungi which includes the insertion of homogenized mycelium (previously grown in liquid medium) in calcium alginate beads [132]. The latter is a highly compatible inoculum method for seedlings raised at commercial nurseries [98] and carries better protection of fungal mycelium for a more extended time [200]. The conditions of different substrates such as peat, compost, and bark compost significantly impact the mycorrhization rates, growth of host seedlings [215], ECM abundance [216, 217], and composition [218]. In solid substrate fermentation, besides the low cost of vermiculite, its bed-type structure provides proper aeration. The ECM cultivation in liquid culture medium requires identifying optimum agitation speed for production of high dry weighted mycelium, maintaining sufficient aeration conditions, and minimizing the shear forces where the agitation speed between 150 and 250 rpm is reported as optimal for mass production of ECM mycelium [219]. Despite ECM submerged cultivation's better performance than solid substrate fermentation, the former technique has limitations of high contamination frequency and ECM fungal poor growth [220]. The ECM-inoculated seedling growth can be positively or negatively impacted by the nursery environmental conditions. The host seedling growth differs when inoculated with single ECM fungal species and inoculated with various multiple species of ECM [221].

ECM fungi have another very essential aspect of invasiveness. The invaded ECM fungi can compete with beneficial native fungi for the resources and promote the co-invasion of non- native trees. This disturbs the natural ecosystem and proves to be harmful to humans in some cases. So introducing non-native ECM fungi in a particular area can emerge into other challenge for researchers regarding their management and eradication [222]. Under restoration practices, introducing exotic plant species can obstruct the native plant establishment and growth and influence several properties of an ecosystem, thus highlighting the need to check alien plant species introduction in various programs [223]. The symbiotic ECM fungi have an essential role in conserving endangered plant species programs that have been ignored in the past. The conserved ex situ endangered tree population on sites other than their native geographical areas does not automatically conserve their native ECM fungal species but rather is found in symbiosis with non-native ECM fungi gained from nurseries where seedlings are raised before field transplantations. It thus highlights the necessity of preventing non-native ECM fungal symbiosis with ex situ conserved endangered trees by introducing the native fungal partners to the latter artificially [224].

2.8 Future Trends and Conclusions

Till now, numerous studies reported the ecological significance of ECM fungi, predominantly their critical role in nutrient cycling of the forest ecosystem and their potent ability to tolerate various environmental stress conditions. Furthermore, the symbiosis of ECM fungi ameliorates the plant nutrient and water uptake from soil and confers them a tolerance against biotic and abiotic stresses. ECM fungi as biofertilizer is highly efficient in replacing chemical fertilizers at commercial nurseries based on ECM-mediated ameliorated host plant nutrient and water uptake from soil. Based on the evidence collected, ECM fungal symbiosis is a powerful biotechnology tool for mediating plant regeneration on lands degraded by anthropogenic or natural disturbances. To establish successful ECM-plant symbiosis for rehabilitation programs, (i) ECM fungus species selection, (ii) compatibility of both symbiotic partners for each other, (iii) ECM colonization rate with host roots, (iv) native or non-native origins of both partners for targeted geographical areas, (v) soil composition and edaphic properties, (vi) ECM fungal inoculum type and inoculation technology, (vii) fungal mycelium exploration type, and (viii) symbiosis induction at nurseries before out-plantation in fields are the mandatory factors to consider which if done carefully, the success chances of restoration programs would enhance. Even after optimizing the best symbiosis parameters for restoration, the major challenge still remains in producing higher ECM fungal inoculum for large-scale restoration programs, which requires more studies and standardizations. The standardization of various parameters discussed above is needed to focus on future studies for better utilization of ECM symbiosis in restoration programs.

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References

1. Curebal I, Efe R, Soykan A, Sonmez S (2015) Impacts of anthropogenic factors on land degradation during the anthropocene in Turkey. J Environ Biol 36(1):51
2. Khosla B, Reddy MS (2014) Mycorrhizal fungi in extreme environments and their impact on plant growth. Kavaka 42:123–130
3. Guala SD, Vega FA, Covelo EF (2010) The dynamics of heavy metals in plant–soil interactions. Ecol Model 221(8):1148–1152
4. Long Z, Huang Y, Zhang W, Shi Z, Yu D, Chen Y et al (2021) Effect of different industrial activities on soil heavy metal pollution, ecological risk, and health risk. Environ Monit Assess 193(1):1–12
5. Oliveira A, Pampulha ME (2006) Effects of long-term heavy metal contamination on soil microbial characteristics. J Biosci Bioeng 102(3):157–161
6. Zhang C, Wang X, Ashraf U, Qiu B, Ali S (2017) Transfer of lead (Pb) in the soil-plant-mealybug-ladybird beetle food chain, a comparison between two host plants. Ecotoxicol Environ Saf 143:289–295
7. Change IC (2014) Mitigation of climate change. Contribution of Working Group III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, 1454. Energy Procedia 147
8. Stocker T (ed) (2014) Climate change 2013: the physical science basis: Working Group I contribution to the Fifth assessment report of the Intergovernmental Panel on Climate Change. Cambridge University Press
9. Zandalinas SI, Balfagón D, Arbona V, Gómez-Cadenas A (2017) Modulation of antioxidant defense system is associated with combined drought and heat stress tolerance in citrus. Front Plant Sci 8:953
10. Millar CI, Stephenson NL (2015) Temperate forest health in an era of emerging megadisturbance. Science 349(6250):823–826
11. Seidl R, Thom D, Kautz M, Martin-Benito D, Peltoniemi M, Vacchiano G et al (2017) Forest disturbances under climate change. Nat Clim Chang 7(6):395–402
12. Miyamoto Y, Danilov AV, Bryanin SV (2021) The dominance of *Suillus* species in ectomycorrhizal fungal communities on *Larix gmelinii* in a post-fire forest in the Russian Far East. Mycorrhiza 31(1):55–66
13. Neary DG, Klopatek CC, DeBano LF, Ffolliott PF (1999) Fire effects on belowground sustainability: a review and synthesis. For Ecol Manag 122(1–2):51–71
14. Smith SE, Read DJ (2010) Mycorrhizal symbiosis. Academic Press
15. Tedersoo L, Smith ME (2013) Lineages of ectomycorrhizal fungi revisited: foraging strategies and novel lineages revealed by sequences from belowground. Fungal Biol Rev 27(3–4):83–99
16. Van der Heijden MG, Martin FM, Selosse MA, Sanders IR (2015) Mycorrhizal ecology and evolution: the past, the present, and the future. New Phytol 205:1406–1423
17. Averill C, Turner BL, Finzi AC (2014) Mycorrhiza-mediated competition between plants and decomposers drives soil carbon storage. Nature 505(7484):543–545
18. Brzostek ER, Dragoni D, Brown ZA, Phillips RP (2015) Mycorrhizal type determines the magnitude and direction of root-induced changes in decomposition in a temperate forest. New Phytol 206(4):1274–1282
19. Gadgil PD, Gadgil RL (1975) Suppression of litter decomposition by mycorrhizal roots of *Pinus radiata*, New Zealand Forest Service
20. Jackson O, Quilliam RS, Stott A, Grant H, Subke JA (2019) Rhizosphere carbon supply accelerates soil organic matter decomposition in the presence of fresh organic substrates. Plant Soil 440(1–2):473–490

21. Kalucka IL, Jagodzinski AM (2016) Successional traits of ectomycorrhizal fungi in forest reclamation after surface mining and agricultural disturbances: a review. Dendrobiology 76: 91–104
22. Sousa NR, Franco AR, Ramos MA, Oliveira RS, Castro PM (2011) Reforestation of burned stands: the effect of ectomycorrhizal fungi on *Pinus pinaster* establishment. Soil Biol Biochem 43(10):2115–2120
23. Gagné A, Jany JL, Bousquet J, Khasa DP (2006) Ectomycorrhizal fungal communities of nursery-inoculated seedlings outplanted on clear-cut sites in northern Alberta. Can J For Res 36(7):1684–1694
24. El-Bashiti TA, El-Kichaoui A, Ajwa AHA (2017) Evaluation the effect of ectomycorrhizal fungi on *Prunus cerasifera* x salicina (Rosaceae) growth compared with chemical and organic fertilizer. Sky J Agric Res 6:41–49
25. Ashkannejhad S, Horton TR (2006) Ectomycorrhizal ecology under primary succession on coastal sand dunes: interactions involving Pinus contorta, suilloid fungi and deer. New Phytol 169(2):345–354
26. Nara K (2006) Pioneer dwarf willow may facilitate tree succession by providing late colonizers with compatible ectomycorrhizal fungi in a primary successional volcanic desert. New Phytol 171(1):187–198
27. Colpaert JV, Wevers JH, Krznaric E, Adriaensen K (2011) How metal-tolerant ecotypes of ectomycorrhizal fungi protect plants from heavy metal pollution. Ann For Sci 68(1):17–24
28. Khullar S, Reddy MS (2019) Cadmium induced glutathione bioaccumulation mediated by γ-glutamylcysteine synthetase in ectomycorrhizal fungus *Hebeloma cylindrosporum*. Biometals 32(1):101–110
29. Prin Y, Ducousso M, Tassin J, Béna G, Jourand P, Dumontet V, et al (2012) Ectotrophic mycorrhizal symbioses are dominant in natural ultramafic forest ecosystems of New Caledonia
30. Aryal P, Meiners SJ, Carlsward BS (2020) Ectomycorrhizae determine chestnut seedling growth and drought response. Agrofor Syst:1–10
31. Smith SE, Read D (2008) Mycorrhizal symbiosis third edition introduction. *Mycorrhizal Symbiosis*:1–9
32. Nannipieri P, Eldor P (2009) The chemical and functional characterization of soil N and its biotic components. Soil Biol Biochem 41(12):2357–2369
33. Condron LM, Turner BL, Cade-Menun BJ (2005) Chemistry and dynamics of soil organic phosphorus. In: Phosphorus: agriculture and the environment, vol 46. pp 87–121
34. Turner BL, Papházy MJ, Haygarth PM, McKelvie ID (2002) Inositol phosphates in the environment. Philos Trans R Soc Lond B Biol Sci 357(1420):449–469
35. Hobbie EA, Horton TR (2007) Evidence that saprotrophic fungi mobilise carbon and mycorrhizal fungi mobilise nitrogen during litter decomposition. New Phytol 173:447–449
36. Hodge A (2017) Accessibility of inorganic and organic nutrients for mycorrhizas. In: Mycorrhizal mediation of soil. Elsevier, pp 129–148
37. Sousa NR, Franco AR, Oliveira RS, Castro PM (2012) Ectomycorrhizal fungi as an alternative to the use of chemical fertilisers in nursery production of *Pinus pinaster*. J Environ Manag 95: S269–S274
38. Melin E, Nilsson H (1957) Transport of C14-labelled photosynthate to the fungal associate of pine mycorrhiza. Sven Bot Tidskr 51:166–186
39. Cairney JWG, Ashford AE, Allaway WG (1989) Distribution of photosynthetically fixed carbon within root systems of *Eucalyptus pilularis* plants ectomycorrhizal with Pisolithus tinctorius. New Phytol 112(4):495–500
40. Wu B, Nara K, Hogetsu T (2002) Spatiotemporal transfer of carbon-14-labelled photosynthate from ectomycorrhizal Pinus densiflora seedlings to extraradical mycelia. Mycorrhiza 12(2): 83–88
41. Smith SE, Read DJ, Last FT (1997) Mycorrhizal symbiosis. Ann Bot 80(5):701

42. Nehls U (2004) Carbohydrates and nitrogen: nutrients and signals in ectomycorrhizas. In: Varma A, Abbott L, Werner D, Hampp R (eds) Plant surface microbiology. Springer, Berlin, pp 373–392
43. Salzer P, Hager A (1991) Sucrose utilization of the ectomycorrhizal fungi *amanita muscaria* and *Hebeloma crustuliniforme* depends on the cell wall-bound invertase activity of their host Picea abies. Bot Acta 104(6):439–445
44. Schaeffer C, Wallenda T, Guttenberger M, HAMPP, R. (1995) Acid invertase in mycorrhizal and non-mycorrhizal roots of Norway spruce (Picea abies [L.] karst.) seedlings. New Phytol 129(3):417–424
45. Nehls U, Hampp R (2000) Carbon allocation in ectomycorrhizas. Physiol Mol Plant Pathol 57(3):95–100
46. Fajardo López M, Dietz S, Grunze N, Bloschies J, Weiß M, Nehls U (2008) The sugar porter gene family of *Laccaria bicolor*: function in ectomycorrhizal symbiosis and soil-growing hyphae. New Phytol 180(2):365–378
47. Nehls U (2008) Mastering ectomycorrhizal symbiosis: the impact of carbohydrates. J Exp Bot 59(5):1097–1108
48. Albarracín MV, Six J, Houlton BZ, Bledsoe CS (2013) A nitrogen fertilization field study of carbon-13 and nitrogen-15 transfers in ectomycorrhizas of Pinus sabiniana. Oecologia 173(4): 1439–1450
49. Hortal S, Plett KL, Plett JM, Cresswell T, Johansen M, Pendall E, Anderson IC (2017) Role of plant–fungal nutrient trading and host control in determining the competitive success of ectomycorrhizal fungi. ISME J 11(12):2666–2676
50. Valtanen K, Eissfeller V, Beyer F, Hertel D, Scheu S, Polle A (2014) Carbon and nitrogen fluxes between beech and their ectomycorrhizal assemblage. Mycorrhiza 24(8):645–650
51. Attiwill PM, Adams MA (1993) Nutrient cycling in forests. New Phytol 124(4):561–582
52. Leinweber P, Kruse J, Baum C, Arcand M, Knight JD, Farrell R et al (2013) Advances in understanding organic nitrogen chemistry in soils using state-of-the-art analytical techniques. Adv Agron 119:83–151
53. Simpson AJ, Song G, Smith E, Lam B, Novotny EH, Hayes MH (2007) Unraveling the structural components of soil humin by use of solution-state nuclear magnetic resonance spectroscopy. Environ Sci Technol 41(3):876–883
54. Nicolás C, Martin-Bertelsen T, Floudas D, Bentzer J, Smits M, Johansson T et al (2019) The soil organic matter decomposition mechanisms in ectomycorrhizal fungi are tuned for liberating soil organic nitrogen. ISME J 13(4):977–988
55. Plett KL, Singan VR, Wang M, Ng V, Grigoriev IV, Martin F et al (2020) Inorganic nitrogen availability alters *Eucalyptus grandis* receptivity to the ectomycorrhizal fungus *Pisolithus albus* but not symbiotic nitrogen transfer. New Phytol 226(1):221–231
56. LeDuc SD, Lilleskov EA, Horton TR, Rothstein DE (2013) Ectomycorrhizal fungal succession coincides with shifts in organic nitrogen availability and canopy closure in post-wildfire jack pine forests. Oecologia 172(1):257–269
57. Zhu H, Guo DC, Dancik BP (1990) Purification and characterization of an extracellular acid proteinase from the ectomycorrhizal fungus *Hebeloma crustuliniforme*. Appl Environ Microbiol 56(4):837–843
58. Nehls U, Bock A, Einig W, Hampp R (2001) Excretion of two proteases by the ectomycorrhizal fungus Amanita muscaria. Plant Cell Environ 24(7):741–747
59. Martin F, Selosse MA (2008) The *Laccaria* genome: a symbiont blueprint decoded. New Phytol:296–310
60. Shah F, Rineau F, Canbäck B, Johansson T, Tunlid A (2013) The molecular components of the extracellular protein-degradation pathways of the ectomycorrhizal fungus *Paxillus involutus*. New Phytol 200(3):875–887
61. Rineau F, Stas J, Nguyen NH, Kuyper TW, Carleer R, Vangronsveld J et al (2016) Ectomycorrhizal fungal protein degradation ability predicted by soil organic nitrogen availability. Appl Environ Microbiol 82(5):1391–1400

62. Jilling A, Keiluweit M, Contosta AR, Frey S, Schimel J, Schnecker J et al (2018) Minerals in the rhizosphere: overlooked mediators of soil nitrogen availability to plants and microbes. Biogeochemistry 139(2):103–122
63. Mikutta R, Turner S, Schippers A, Gentsch N, Meyer-Stüve S, Condron LM et al (2019) Microbial and abiotic controls on mineral-associated organic matter in soil profiles along an ecosystem gradient. Sci Rep 9(1):1–9
64. Keiluweit M, Bougoure JJ, Nico PS, Pett-Ridge J, Weber PK, Kleber M (2015) Mineral protection of soil carbon counteracted by root exudates. Nat Clim Chang 5(6):588–595
65. Mikutta R, Kleber M, Torn MS, Jahn R (2006) Stabilization of soil organic matter: association with minerals or chemical recalcitrance? Biogeochemistry 77(1):25–56
66. Wang T, Tian Z, Tunlid A, Persson P (2020) Nitrogen acquisition from mineral-associated proteins by an ectomycorrhizal fungus. New Phytol 228(2):697–711
67. Schmalenberger A, Duran AL, Bray AW, Bridge J, Bonneville S, Benning LG et al (2015) Oxalate secretion by ectomycorrhizal *Paxillus involutus* is mineral-specific and controls calcium weathering from minerals. Sci Rep 5(1):1–14
68. Bills GF, Gloer JB (2016) Biologically active secondary metabolites from the fungi. Microbiol Spectr 4(6):4–6
69. Feller BE, Kellis JT Jr, Cascão-Pereira LG, Robertson CR, Frank CW (2011) Interfacial biocatalysis on charged and immobilized substrates: the roles of enzyme and substrate surface charge. Langmuir 27(1):250–263
70. Rosling A, Midgley MG, Cheeke T, Urbina H, Fransson P, Phillips RP (2016) Phosphorus cycling in deciduous forest soil differs between stands dominated by ecto-and arbuscular mycorrhizal trees. New Phytol 209(3):1184–1195
71. Yang X, Post WM (2011) Phosphorus transformations as a function of pedogenesis: a synthesis of soil phosphorus data using Hedley fractionation method. Biogeosciences 8(10): 2907–2916
72. Liu X, Burslem DF, Taylor JD, Taylor AF, Khoo E, Majalap-Lee N et al (2018) Partitioning of soil phosphorus among arbuscular and ectomycorrhizal trees in tropical and subtropical forests. Ecol Lett 21(5):713–723
73. Ruess RW, Swanson MM, Kielland K, McFarland JW, Olson KD, Taylor DL (2019) Phosphorus mobilizing enzymes of alnus-associated ectomycorrhizal fungi in an Alaskan boreal floodplain. Forests 10(7):554
74. Joner EJ, Jakobsen I (1995) Growth and extracellular phosphatase activity of arbuscular mycorrhizal hyphae as influenced by soil organic matter. Soil Biol Biochem 27(9):1153–1159
75. Plassard C, Dell B (2010) Phosphorus nutrition of mycorrhizal trees. Tree Physiol 30(9): 1129–1139
76. Phillips RP, Brzostek E, Midgley MG (2013) The mycorrhizal-associated nutrient economy: a new framework for predicting carbon–nutrient couplings in temperate forests. New Phytol 199(1):41–51
77. Turner BL, Condron LM, Richardson SJ, Peltzer DA, Allison VJ (2007) Soil organic phosphorus transformations during pedogenesis. Ecosystems 10(7):1166–1181
78. Colpaert JV, Van Laere A, Van Tichelen KK, Van Assche JA (1997) The use of inositol hexaphosphate as a phosphorus source by mycorrhizal and non-mycorrhizal Scots Pine (*Pinus sylvestris*). Funct Ecol 11(4):407–415
79. Kroehler CJ, Linkins AE (1988) The root surface phosphatases of *Eriophorum vaginatum*: effects of temperature, pH, substrate concentration and inorganic phosphorus. Plant Soil 105(1):3–10
80. Müller K, Kubsch N, Marhan S, Mayer-Gruner P, Nassal P, Schneider D et al (2020) Saprotrophic and ectomycorrhizal fungi contribute differentially to organic P mobilization in beech-dominated forest ecosystems. Front For Glob Change 3:47
81. Becquer A, Garcia K, Plassard C (2018) HcPT1. 2 participates in Pi acquisition in *Hebeloma cylindrosporum* external hyphae of ectomycorrhizas under high and low phosphate conditions. Plant Signal Behav 13(10):e1525997

82. Garcia K, Haider MZ, Delteil A, Corratgé-Faillie C, Conéjero G, Tatry MV et al (2013) Promoter-dependent expression of the fungal transporter HcPT1. 1 under pi shortage and its spatial localization in ectomycorrhiza. Fungal Genet Biol 58:53–61
83. Casieri L, Lahmidi NA, Doidy J, Veneault-Fourrey C, Migeon A, Bonneau L et al (2013) Biotrophic transportome in mutualistic plant–fungal interactions. Mycorrhiza 23(8):597–625
84. Zheng R, Wang J, Liu M, Duan G, Gao X, Bai S, Han Y (2016) Molecular cloning and functional analysis of two phosphate transporter genes from *Rhizopogon luteolus* and *Leucocortinarius bulbiger*, two ectomycorrhizal fungi of Pinus tabulaeformis. Mycorrhiza 26(7):633–644
85. Ali MA, Louche J, Legname E, Duchemin M, Plassard C (2009) Pinus pinaster seedlings and their fungal symbionts show high plasticity in phosphorus acquisition in acidic soils. Tree Physiol 29(12):1587–1597
86. Alvarez M, Huygens D, Diaz LM, Villanueva CA, Heyser W, Boeckx P (2012) The spatial distribution of acid phosphatase activity in ectomycorrhizal tissues depends on soil fertility and morphotype, and relates to host plant phosphorus uptake. Plant Cell Environ 35(1):126–135
87. Gray L, Kernaghan G (2020) Fungal succession during the decomposition of ectomycorrhizal fine roots. Microb Ecol 79(2):271–284
88. Langley JA, Hungate BA (2003) Mycorrhizal controls on belowground litter quality. Ecology 84(9):2302–2312
89. Brabcová V, Nováková M, Davidová A, Baldrian P (2016) Dead fungal mycelium in forest soil represents a decomposition hotspot and a habitat for a specific microbial community. New Phytol 210(4):1369–1381
90. Brabcová V, Štursová M, Baldrian P (2018) Nutrient content affects the turnover of fungal biomass in forest topsoil and the composition of associated microbial communities. Soil Biol Biochem 118:187–198
91. Kaneda S, Kaneko N (2004) The feeding preference of a collembolan (*Folsomia candida* Willem) on ectomycorrhiza (Pisolithus tinctorius (Pers.)) varies with mycelial growth condition and vitality. Appl Soil Ecol 27(1):1–5
92. Thimm T, Hoffmann A, Borkott H, Munch JC, Tebbe CC (1998) The gut of the soil microarthropod *Folsomia candida* (Collembola) is a frequently changeable but selective habitat and a vector for microorganisms. Appl Environ Microbiol 64(7):2660–2669
93. Butler MJ, Day AW (1998) Destruction of fungal melanins by ligninases of Phanerochaete chrysosporium and other white rot fungi. Int J Plant Sci 159(6):989–995
94. Fernandez CW, Koide RT (2012) The role of chitin in the decomposition of ectomycorrhizal fungal litter. Ecology 93(1):24–28
95. Šnajdr J, Valášková V, Merhautová V, Herinková J, Cajthaml T, Baldrian P (2008) Spatial variability of enzyme activities and microbial biomass in the upper layers of Quercus petraea forest soil. Soil Biol Biochem 40(9):2068–2075
96. Solly EF, Schöning I, Boch S, Kandeler E, Marhan S, Michalzik B et al (2014) Factors controlling decomposition rates of fine root litter in temperate forests and grasslands. Plant Soil 382(1–2):203–218
97. Mohan V, Nivea R, Menon S (2015) Evaluation of ectomycorrhizal fungi as potential bio-control agents against selected plant pathogenic fungi. JAIR 3(9):408–412
98. Costa LSD, Grazziotti PH, Silva AC, Fonseca AJ, Gomes ÂLF, Grazziotti DCFS, Rossi MJ (2019) Alginate gel entrapped ectomycorrhizal inoculum promoted growth of cuttings of Eucalyptus clones under nursery conditions. Can J For Res 48(8):978–985
99. Yang N, Zavišić A, Pena R, Polle A (2016) Phenology, photosynthesis, and phosphorus in European beech (Fagus sylvatica L.) in two forest soils with contrasting P contents. J Plant Nutr Soil Sci 179(2):151–158
100. Zavišić A, Yang N, Marhan S, Kandeler E, Polle A (2018) Forest soil phosphorus resources and fertilization affect ectomycorrhizal community composition, beech P uptake efficiency, and photosynthesis. Front Plant Sci 9:463

101. Gerasko T, Pyda S (2021) Effect of inoculation with symbiotic endo-and ectomycorrhizal fungi on content of basic mineral nutrients in sweet cherry leaves
102. Yousefshahi B, Bazgir M (2017) Inoculation process for brants oak (*Quercus* brantii Lindl.) seedling with ectomycorrhizal fungi in hydroponic culture condition. Iran J For Poplar Res 25(4):675–686
103. Verma B, Reddy MS (2020) Biochar augmentation improves ectomycorrhizal colonisation, plant growth and soil fertility. Soil Res 58(7):673–682
104. Frank HE, Garcia K (2021) Benefits provided by four ectomycorrhizal fungi to Pinus taeda under different external potassium availabilities. Mycorrhiza 31(6):755–766
105. Zhang L, Zhou G, Liu J, Li L, Wang S (2012) Using ectomycorrhizal inocula to increase slash pine (*Pinus elliottii*) growth in Southern China. Afr J Microbiol Res 6(41):6936–6940
106. Gandini AMM, Grazziotti PH, Rossi MJ, Grazziotti DCFS, Gandini EMM, Silva EDB, Ragonezi C (2015) Growth and nutrition of eucalypt rooted cuttings promoted by ectomycorrhizal fungi in commercial nurseries. Rev Bras Ciênc Solo 39:1554–1565
107. Oliveira RS, Franco AR, Castro PM (2012) Combined use of Pinus pinaster plus and inoculation with selected ectomycorrhizal fungi as an ecotechnology to improve plant performance. Ecol Eng 43:95–103
108. Sebastiana M, Pereira VT, Alcântara A, Pais MS, Silva AB (2013) Ectomycorrhizal inoculation with *Pisolithus tinctorius* increases the performance of *Quercus suber* L.(cork oak) nursery and field seedlings. New For 44(6):937–949
109. Lehto T, Zwiazek JJ (2011) Ectomycorrhizas and water relations of trees: a review. Mycorrhiza 21(2):71–90
110. Yin D, Qi J, Deng J, Deng X (2017) Effects of ectomycorrhizal cooperating with exogenous calcium on *Pinus sylvestris* var. *mongolica* growth. China Environ Sci 37(6):2295–2304
111. Bzdyk RM, Olchowik J, Studnicki M, Oszako T, Sikora K, Szmidla H, Hilszczańska D (2018) The impact of effective microorganisms (EM) and organic and mineral fertilizers on the growth and mycorrhizal colonization of Fagus sylvatica and Quercus robur seedlings in a bare-root nursery experiment. Forests 9(10):597
112. Wang L, Katzensteiner K, Schume H, Van Loo M, Godbold DL (2016) Potassium fertilization affects the distribution of fine roots but does not change ectomycorrhizal community structure. Ann For Sci 73(3):691–702
113. Rudawska M, Leski T (2021) Ectomycorrhizal fungal assemblages of nursery-grown scots pine are influenced by age of the seedlings. Forests 12(2):134
114. Trocha LK, Rudawska M, Leski T, Dabert M (2006) Genetic diversity of naturally established ectomycorrhizal fungi on Norway spruce seedlings under nursery conditions. Microb Ecol 52(3):418–425
115. Clausing S, Likulunga LE, Janz D, Feng HY, Schneider D, Daniel R et al (2021) Impact of nitrogen and phosphorus addition on resident soil and root mycobiomes in beech forests. Biol Fertil Soils 57(8):1031–1052
116. Bahr A, Ellström M, Bergh J, Wallander H (2015) Nitrogen leaching and ectomycorrhizal nitrogen retention capacity in a Norway spruce forest fertilized with nitrogen and phosphorus. Plant Soil 390(1):323–335
117. Klavina D, Pennanen T, Gaitnieks T, Velmala S, Lazdins A, Lazdina D, Menkis A (2016) The ectomycorrhizal community of conifer stands on peat soils 12 years after fertilization with wood ash. Mycorrhiza 26(2):153–160
118. Rudawska M, Leski T, Aučina A, Karliński L, Skridaila A, Ryliškis D (2017) Forest litter amendment during nursery stage influence field performance and ectomycorrhizal community of Scots pine (Pinus sylvestris L.) seedlings outplanted on four different sites. For Ecol Manag 395:104–114
119. Augusto L, Bakker MR, Meredieu C (2008) Wood ash applications to temperate forest ecosystems—potential benefits and drawbacks. Plant Soil 306(1):181–198

120. Ericsson T (1995) Growth and shoot: root ratio of seedlings in relation to nutrient availability. In: Nutrient uptake and cycling in forest ecosystems. Springer, Dordrecht, pp 205–214
121. Berdén M, Nilsson SI, Nyman P (1997) Ion leaching before and after clear-cutting in a Norway spruce stand—effects of long-term application of ammonium nitrate and superphosphate. Water Air Soil Pollut 93(1):1–26
122. Akselsson C, Belyazid S, Hellsten S, Klarqvist M, Pihl-Karlsson G, Karlsson PE, Lundin L (2010) Assessing the risk of N leaching from forest soils across a steep N deposition gradient in Sweden. Environ Pollut 158(12):3588–3595
123. Högberg MN, Skyllberg U, Högberg P, Knicker H (2020) Does ectomycorrhiza have a universal key role in the formation of soil organic matter in boreal forests? Soil Biol Biochem 140:107635
124. Kottke I, Qian XM, Pritsch K, Haug I, Oberwinkler F (1998) Xerocomus badius–Picea abies, an ectomycorrhiza of high activity and element storage capacity in acidic soil. Mycorrhiza 7(5):267–275
125. Iwański M, Rudawska M, Leski T (2006) Mycorrhizal associations of nursery grown Scots pine (Pinus sylvestris L.) seedlings in Poland. Ann For Sci 63(7):715–723
126. Leski T, Rudawska M, Aučina A (2008) The ectomycorrhizal status of European larch (*Larix decidua* Mill.) seedlings from bare-root forest nurseries. For Ecol Manag 256(12):2136–2144
127. Castrillón M, León JD, Carvajal D, Osorio NW (2015) Effectiveness of single and combined ectomycorrhizal inocula on three species of *Pinus* at nursery. Commun Soil Sci Plant Anal 46(2):169–179
128. García FG, Valdés RC, Sanchez Peña S, González Morales S, Mendoza Villarreal R (2019) Improved parameters of *Pinus greggii* seedling growth and health after inoculation with ectomycorrhizal fungi. South For 81(1):23–30
129. Reddy MS, Natarajan K (1997) Coinoculation efficacy of ectomycorrhizal fungi on Pinus patula seedlings in a nursery. Mycorrhiza 7(3):133–138
130. Lofgren L, Nguyen NH, Kennedy PG (2018) Ectomycorrhizal host specificity in a changing world: can legacy effects explain anomalous current associations? New Phytol 220(4): 1273–1284
131. Murat C, Zampieri E, Vizzini A, Bonfante P (2008) Is the Perigord black truffle threatened by an invasive species? We dreaded it and it has happened! New Phytol 178(4):699–702
132. Rossi MJ, Furigo A Jr, Oliveira VL (2007) Inoculant production of ectomycorrhizal fungi by solid and submerged fermentations. Food Technol Biotechnol 45(3):277–286
133. Anjum SA, Ashraf U, Zohaib A, Tanveer M, Naeem M, Ali I et al (2017) Growth and development responses of crop plants under drought stress: a review. Zemdirbyste 104(3): 267–276
134. Ullah A, Mushtaq H, Fahad S, Shah A, Chaudhary HJ (2017) Plant growth promoting potential of bacterial endophytes in novel association with *Olea ferruginea* and *Withania coagulans*. Microbiology 86(1):119–127
135. Vayssières A, Pěnčík A, Felten J, Kohler A, Ljung K, Martin F, Legué V (2015) Development of the poplar-Laccaria bicolor ectomycorrhiza modifies root auxin metabolism, signaling, and response. Plant Physiol 169(1):890–902
136. García de Jalón L, Limousin JM, Richard F, Gessler A, Peter M, Hättenschwiler S, Milcu A (2020) Microhabitat and ectomycorrhizal effects on the establishment, growth and survival of Quercus ilex L. seedlings under drought. PLoS One 15(6):e0229807
137. Calvo-Polanco M, Armada E, Zamarreño AM, García-Mina JM, Aroca R (2019) Local root ABA/cytokinin status and aquaporins regulate poplar responses to mild drought stress independently of the ectomycorrhizal fungus *Laccaria bicolor*. J Exp Bot 70(21):6437–6446
138. Zhu C, Schraut D, Hartung W, Schäffner AR (2005) Differential responses of maize MIP genes to salt stress and ABA. J Exp Bot 56(421):2971–2981

139. Ruiz-Lozano JM, del Mar AM, Bárzana G, Vernieri P, Aroca R (2009) Exogenous ABA accentuates the differences in root hydraulic properties between mycorrhizal and non mycorrhizal maize plants through regulation of PIP aquaporins. Plant Mol Biol 70(5):565–579
140. Allen MF (2007) Mycorrhizal fungi: highways for water and nutrients in arid soils. Vadose Zone J 6(2):291–297
141. Hirschi KD (2004) The calcium conundrum. Both versatile nutrient and specific signal. Plant Physiol 136(1):2438–2442
142. Johansson I, Larsson C, Ek B, Kjellbom P (1996) The major integral proteins of spinach leaf plasma membranes are putative aquaporins and are phosphorylated in response to Ca^{2+} and apoplastic water potential. Plant Cell 8(7):1181–1191
143. Shinozaki K, Yamaguchi-Shinozaki K (1997) Gene expression and signal transduction in water-stress response. Plant Physiol 115(2):327
144. Yin D, Deng X, Song R (2016) Synergistic effects between *Suillus luteus* and *Trichoderma virens* on growth of Korean spruce seedlings and drought resistance of Scotch pine seedlings. J For Res 27(1):193–201
145. Wang J, Zhang H, Gao J, Zhang Y, Liu Y, Tang M (2021) Effects of ectomycorrhizal fungi (Suillus variegatus) on the growth, hydraulic function, and non-structural carbohydrates of *Pinus tabulaeformis* under drought stress. BMC Plant Biol 21(1):1–13
146. Alvarez M, Huygens D, Olivares E, Saavedra I, Alberdi M, Valenzuela E (2009) Ectomycorrhizal fungi enhance nitrogen and phosphorus nutrition of *Nothofagus dombeyi* under drought conditions by regulating assimilative enzyme activities. Physiol Plant 136(4): 426–436
147. Yin D, Wang H, Qi J (2021) The enhancement effect of calcium ions on ectomycorrhizal fungi-mediated drought resistance in *Pinus sylvestris* var. *mongolica*. J Plant Growth Regul 40(4):1389–1399
148. Li Y, Zhang T, Zhou Y, Zou X, Yin Y, Li H et al (2021) Ectomycorrhizal symbioses increase soil calcium availability and water use efficiency of *Quercus acutissima* seedlings under drought stress. Eur J For Res 140:1039–1048
149. Sebastiana M, Duarte B, Monteiro F, Malhó R, Caçador I, Matos AR (2019) The leaf lipid composition of ectomycorrhizal oak plants shows a drought-tolerance signature. Plant Physiol Biochem 144:157–165
150. Alberdi M, Alvarez M, Valenzuela E, Godoy R, Olivares E, Barrientos M (2007) Response to water deficit of *Nothofagus dombeyi* plants inoculated with a specific (*Descolea Antarctica* sing) and non-specific (*Pisolithus tinctorius* (Pers.) Coker & Couch) ectomycorrhizal fungi. Rev Chil Hist Nat 80(4):479–491
151. Luo YY (1997) Oxidation and dissolution of lead in chlorinated drinking water. Adv Environ Res 1:84–97
152. Vaclavikova M, Gallios GP, Hredzak S, Jakabsky S (2008) Removal of arsenic from water streams: an overview of available techniques. Clean Techn Environ Policy 10(1):89–95
153. Dong B, Zhang R, Gan Y, Cai L, Freidenreich A, Wang K et al (2019) Multiple methods for the identification of heavy metal sources in cropland soils from a resource-based region. Sci Total Environ 651:3127–3138
154. Argun ME, Dursun S, Ozdemir C, Karatas M (2007) Heavy metal adsorption by modified oak sawdust: thermodynamics and kinetics. J Hazard Mater 141(1):77–85
155. Duruibe JO, Ogwuegbu MOC, Egwurugwu JN (2007) Heavy metal pollution and human biotoxic effects. Int J Phys Sci 2(5):112–118
156. Odum HT (ed) (2016) Heavy metals in the environment: using wetlands for their removal. CRC Press
157. Adriaensen K, Vangronsveld J, Colpaert JV (2006) Zinc-tolerant Suillus bovinus improves growth of Zn-exposed *Pinus sylvestris* seedlings. Mycorrhiza 16(8):553–558
158. Branco S, Ree RH (2010) Serpentine soils do not limit mycorrhizal fungal diversity. PLoS One 5(7):e11757

159. Moser AM, Frank JL, D'Allura JA, Southworth D (2009) Ectomycorrhizal communities of *Quercus garryana* are similar on serpentine and nonserpentine soils. Plant Soil 315(1): 185–194
160. Gonçalves SC, Martins-Loução MA, Freitas H (2009) Evidence of adaptive tolerance to nickel in isolates of *Cenococcum geophilum* from serpentine soils. Mycorrhiza 19(4):221–230
161. Ruytinx J, Nguyen H, Van Hees M, Op De Beeck M, Vangronsveld J, Carleer R et al (2013) Zinc export results in adaptive zinc tolerance in the ectomycorrhizal basidiomycete Suillus bovinus. Metallomics 5(9):1225–1233
162. Ray P, Adholeya A (2009) Correlation between organic acid exudation and metal uptake by ectomycorrhizal fungi grown on pond ash in vitro. Biometals 22(2):275
163. Bellion M, Courbot M, Jacob C, Blaudez D, Chalot M (2006) Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi. FEMS Microbiol Lett 254(2):173–181
164. Jentschke G, Godbold DL (2000) Metal toxicity and ectomycorrhizas. Physiol Plant 109(2): 107–116
165. Majorel C, Hannibal L, Ducousso M, Lebrun M, Jourand P (2014) Evidence of nickel (Ni) efflux in Ni-tolerant ectomycorrhizal *Pisolithus albus* isolated from ultramafic soil. Environ Microbiol Rep 6(5):510–518
166. Margoshes M, Vallee BL (1957) A cadmium protein from equine kidney cortex. J Am Chem Soc 79(17):4813–4814
167. Cobbett C, Goldsbrough P (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu Rev Plant Biol 53(1):159–182
168. Zhigang A, Cuijie L, Yuangang Z, Yejie D, Wachter A, Gromes R, Rausch T (2006) Expression of BjMT2, a metallothionein 2 from *Brassica juncea*, increases copper and cadmium tolerance in *Escherichia coli* and *Arabidopsis thaliana*, but inhibits root elongation in *Arabidopsis thaliana* seedlings. J Exp Bot 57(14):3575–3582
169. Zhu W, Zhao DX, Miao Q, Xue TT, Li XZ, Zheng CC (2009) *Arabidopsis thaliana* metallothionein, AtMT2a, mediates ROS balance during oxidative stress. J Plant Biol 52(6): 585–592
170. Reddy MS, Prasanna L, Marmeisse R, Fraissinet-Tachet L (2014) Differential expression of metallothioneins in response to heavy metals and their involvement in metal tolerance in the symbiotic basidiomycete Laccaria bicolor. Microbiology 160(10):2235–2242
171. Nguyen H, Rineau F, Vangronsveld J, Cuypers A, Colpaert JV, Ruytinx J (2017) A novel, highly conserved metallothionein family in basidiomycete fungi and characterization of two representative SlMTa and SlMTb genes in the ectomycorrhizal fungus Suillus luteus. Environ Microbiol 19(7):2577–2587
172. Reddy MS, Kour M, Aggarwal S, Ahuja S, Marmeisse R, Fraissinet-Tachet L (2016) Metal induction of a Pisolithus albus metallothionein and its potential involvement in heavy metal tolerance during mycorrhizal symbiosis. Environ Microbiol 18(8):2446–2454
173. Hložková K, Matěnová M, Žáčková P, Strnad H, Hršelová H, Hroudová M, Kotrba P (2016) Characterization of three distinct metallothionein genes of the Ag-hyperaccumulating ectomycorrhizal fungus *Amanita strobiliformis*. Fungal Biol 120(3):358–369
174. Pócsi I, Prade RA, Penninckx MJ (2004) Glutathione, altruistic metabolite in fungi. Adv Microb Physiol 49:1–76
175. Hematy K, Lim M, Cherk C, Bednarek P, Piślewska-Bednarek M, Sanchez-Rodriguez C et al (2019) Arabidopsis phytochelatin synthase 1, but not phytochelatin synthesis, functions in extracellular defense against multiple fungal pathogens. BioRxiv 568113
176. Dučić T, Parladé J, Polle A (2008) The influence of the ectomycorrhizal fungus *Rhizopogon subareolatus* on growth and nutrient element localisation in two varieties of Douglas fir (Pseudotsuga menziesii var. menziesii and var. glauca) in response to manganese stress. Mycorrhiza 18(5):227–239

177. Langer I, Santner J, Krpata D, Fitz WJ, Wenzel WW, Schweiger PF (2012) Ectomycorrhizal impact on Zn accumulation of *Populus tremula* L. grown in metalliferous soil with increasing levels of Zn concentration. Plant Soil 355(1):283–297
178. Hachani C, Lamhamedi MS, Cameselle C, Gouveia S, Zine El Abidine A, Khasa DP, Béjaoui Z (2020) Effects of ectomycorrhizal fungi and heavy metals (Pb, Zn, and Cd) on growth and mineral nutrition of Pinus halepensis seedlings in North Africa. Microorganisms 8(12):2033
179. Tang Y, Shi L, Zhong K, Shen Z, Chen Y (2019) Ectomycorrhizal fungi may not act as a barrier inhibiting host plant absorption of heavy metals. Chemosphere 215:115–123
180. Wen Z, Shi L, Tang Y, Shen Z, Xia Y, Chen Y (2017) Effects of Pisolithus tinctorius and Cenococcum geophilum inoculation on pine in copper-contaminated soil to enhance phytoremediation. Int J Phytoremediation 19(4):387–394
181. Ngugi MR, Fechner N, Neldner VJ, Dennis PG (2020) Successional dynamics of soil fungal diversity along a restoration chronosequence post-coal mining. Restor Ecol 28(3):543–552
182. Neldner VJ, Ngugi MR (2017) Establishment of woody species across 26 years of revegetation on a Queensland coal mine. Ecol Manage Restor 18(1):75–78
183. Gebhardt S, Neubert K, Wöllecke J, Münzenberger B, Hüttl RF (2007) Ectomycorrhiza communities of red oak (Quercus rubra L.) of different age in the Lusatian lignite mining district, East Germany. Mycorrhiza 17(4):279–290
184. Pressler Y, Moore JC, Cotrufo MF (2019) Belowground community responses to fire: meta-analysis reveals contrasting responses of soil microorganisms and mesofauna. Oikos 128(3): 309–327
185. Yang T, Tedersoo L, Lin X, Fitzpatrick MC, Jia Y, Liu X et al (2020) Distinct fungal successional trajectories following wildfire between soil horizons in a cold-temperate forest. New Phytol 227(2):572–587
186. Dahlberg A (2002) Effects of fire on ectomycorrhizal fungi in Fennoscandian boreal forests. Silva Fennica 36(1):69–80
187. Grogan P, Baar J, Bruns TD (2000) Below-ground ectomycorrhizal community structure in a recently burned bishop pine forest. J Ecol 88(6):1051–1062
188. Knicker H (2007) How does fire affect the nature and stability of soil organic nitrogen and carbon? A review. Biogeochemistry 85(1):91–118
189. Glassman SI, Levine CR, DiRocco AM, Battles JJ, Bruns TD (2016) Ectomycorrhizal fungal spore bank recovery after a severe forest fire: some like it hot. ISME J 10(5):1228–1239
190. Burke DJ, Smemo KA, Hewins CR (2014) Ectomycorrhizal fungi isolated from old-growth northern hardwood forest display variability in extracellular enzyme activity in the presence of plant litter. Soil Biol Biochem 68:219–222
191. Buée M, Vairelles D, Garbaye J (2005) Year-round monitoring of diversity and potential metabolic activity of the ectomycorrhizal community in a beech (Fagus sylvatica) forest subjected to two thinning regimes. Mycorrhiza 15(4):235–245
192. Mosca E, Montecchio L, Scattolin L, Garbaye J (2007) Enzymatic activities of three ectomycorrhizal types of *Quercus robur* L. in relation to tree decline and thinning. Soil Biol Biochem 39(11):2897–2904
193. Baar J, Horton TR, Kretzer AM, Bruns TD (1999) Mycorrhizal colonization of Pinus muricata from resistant propagules after a stand-replacing wildfire. New Phytol 143(2):409–418
194. Taylor DL, Bruns TD (1999) Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: minimal overlap between the mature forest and resistant propagule communities. Mol Ecol 8(11):1837–1850
195. Conjeaud C, Scheromm P, Mousain D (1996) Effects of phosphorus and ectomycorrhiza on maritime pine seedlings (Pinus pinaster). New Phytol 133(2):345–351
196. Kipfer T, Moser B, Egli S, Wohlgemuth T, Ghazoul J (2011) Ectomycorrhiza succession patterns in *Pinus sylvestris* forests after stand-replacing fire in the Central Alps. Oecologia 167(1):219–228

197. Pulido-Chavez MF, Alvarado EC, DeLuca TH, Edmonds RL, Glassman SI (2021) High-severity wildfire reduces richness and alters composition of ectomycorrhizal fungi in low-severity adapted ponderosa pine forests. For Ecol Manag 485:118923
198. Sun H, Santalahti M, Pumpanen J, Köster K, Berninger F, Raffaello T et al (2015) Fungal community shifts in structure and function across a boreal forest fire chronosequence. Appl Environ Microbiol 81(22):7869–7880
199. Franco AR, Sousa NR, Ramos MA, Oliveira RS, Castro PM (2014) Diversity and persistence of ectomycorrhizal fungi and their effect on nursery-inoculated Pinus pinaster in a Post-fire plantation in Northern Portugal. Microb Ecol 68(4):761–772
200. Repáč I (2011) Ectomycorrhizal inoculum and inoculation techniques. In: Diversity and biotechnology of ectomycorrhizae. Springer, Berlin, pp 43–63
201. Rudawska M, Leski T, Trocha LK, Gornowicz R (2006) Ectomycorrhizal status of Norway spruce seedlings from bare-root forest nurseries. For Ecol Manage 236(2–3):375–384
202. Taniguchi T, Yuzawa T, HuiPing M, Yamamoto F, Yamanaka N (2021) Plantation soil inoculation combined with straw checkerboard barriers enhances ectomycorrhizal colonization and subsequent growth of nursery grown *Pinus tabulaeformis* seedlings in a dryland. Ecol Eng 163:106191
203. Wubs EJ, Van der Putten WH, Bosch M, Bezemer TM (2016) Soil inoculation steers restoration of terrestrial ecosystems. Nat Plants 2(8):1–5
204. Restrepo-Llano M, Osorio NW, León JD (2014) Assessment of the effectiveness of ectomycorrhizal inocula to promote growth and root ectomycorrhizal colonization in *Pinus patula* seedlings using the most probable number technique. Appl Environ Soil Sci 2:1–6
205. Restrepo-Llano MF, Osorio-Vega NW, León-Peláez JD (2018) Plant growth response of Pinus patula and *P maximinoi* seedlings at nursery to three types of ectomycorrhizal inocula. Appl Environ Soil Sci 2018
206. Chen YL, Kang LH, Dell B (2006) Inoculation of *Eucalyptus urophylla* with spores of *Scleroderma* in a nursery in South China: comparison of field soil and potting mix. For Ecol Manag 222(1–3):439–449
207. Castellano MA, Molina R (1989) The container tree nursery manual-volume 5: the biological component: nursery pests and mycorrhizae. US Department of Agriculture Forest Service. Agriculture Handbook 674
208. Kipfer T, Wohlgemuth T, van der Heijden MG, Ghazoul J, Egli S (2012) Growth response of drought-stressed *Pinus sylvestris* seedlings to single-and multi-species inoculation with ectomycorrhizal fungi. PLoS One 7(4):e35275
209. Moreira FDS, Siqueira JO (2006) Microbiologia e bioquımica do solo. Ufla, Lavras
210. Marx DH, Ruehle JL, Cordell CE (1991) 17 methods for studying nursery and field response of trees to specific ectomycorrhiza. Methods Microbiol 23:383–411
211. Cline ML, Patrick Reid CP (1982) Seed source and mycorrhizal fungus effects on growth of containerized *Pinus contorta* and *Pinus ponderosa* seedlings. For Sci 28(2):237–250
212. Gange AC, Gane DR, Chen Y, Gong M (2005) Dual colonization of Eucalyptus urophylla ST Blake by arbuscular and ectomycorrhizal fungi affects levels of insect herbivore attack. Agric For Entomol 7(3):253–263
213. Alves J, Souza OD, Podlech P, Giachini A, de Oliveira VL (2001) Efeito de inoculante ectomicorrizico por fermentacao semi-solida sobre o crescimento de Eucalyptus dunnii Maiden
214. Vuorinen I, Hamberg L, Müller M, Seiskari P, Pennanen T (2015) Development of growth media for solid substrate propagation of ectomycorrhizal fungi for inoculation of Norway spruce (Picea abies) seedlings. Mycorrhiza 25(4):311–324
215. Repáč I (2007) Ectomycorrhiza formation and growth of Picea abies seedlings inoculated with alginate-bead fungal inoculum in peat and bark compost substrates. Forestry 80(5):517–530
216. Baar J, Elferink MO (1996) Ectomycorrhizal development on Scots pine (*Pinus sylvestris* L.) seedlings in different soils. Plant Soil 179(2):287–292

217. Rincón A, Parladé J, Pera J (2005) Effects of ectomycorrhizal inoculation and the type of substrate on mycorrhization, growth and nutrition of containerised *Pinus pinea* L. seedlings produced in a commercial nursery. Ann For Sci 62(8):817–822
218. Johnson NC, Graham JH, Smith FA (1997) Functioning of mycorrhizal associations along the mutualism–parasitism continuum. New Phytol 135(4):575–585
219. da Costa ECS, dos Santos LL, Grazziotti PH, da Costa LS, Silva CS, de Vasconcelos Ramires R, de Abreu CM (2020) Inoculant production of *Pisolithus* sp. in submerged culture under agitation. Afr J Agric Res 16(5):746–751
220. Charya LS, Garg S (2019) Advances in methods and practices of ectomycorrhizal research. In: Advances in biological science research. Academic Press, pp 303–325
221. Araújo GC, Sousa NR, Ramos MA, Vega AL, Castro PM (2018) Performance of *Quercus suber* L. at nursery stage—application of two bio-inoculants under two distinct environments. Ann For Sci 75(1):1–12
222. Dickie IA, Nuñez MA, Pringle A, Lebel T, Tourtellot SG, Johnston PR (2016) Towards management of invasive ectomycorrhizal fungi. Biol Invasions 18(12):3383–3395
223. Weidlich EW, Flórido FG, Sorrini TB, Brancalion PH (2020) Controlling invasive plant species in ecological restoration: a global review. J Appl Ecol 57(9):1806–1817
224. Sugiyama Y, Murata M, Kanetani S, Nara K (2019) Towards the conservation of ectomycorrhizal fungi on endangered trees: native fungal species on *Pinus amamiana* are rarely conserved in trees planted ex situ. Mycorrhiza 29(3):195–205

Fungal Ministrations in Soil Detoxification, Building, and Health Restoration

3

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Abstract

Soil health refers to its function as a living ecosystem. Food demand for the growing population necessitates boosting of crop productivity. Soil health and crop productivity are closely linked. Various chemicals are often added to the soil at higher doses in an effort to increase productivity, which in the long term deteriorates soil quality. The quality of soil is determined by the availability of essential nutrients. Healthy soil is vital for vibrant microbial diversity. An extensive range of microbes live in soil, which have a participatory contribution in modulating soil properties. The primary role of fungi in the soil ecosystem is decomposition, breakdown of complex substances, plant growth promotion, and biocontrol. Fungi have been extensively studied at pilot and field scale. Employing fungi for biocontrol is also a well-researched area and has a good representation of products in the market. The fungal role in plant growth promotion is relatively an emerging area. This chapter focuses on the role of fungi in defining various functions that impact soil quality. A detailed analysis of the assimilatory actions, dissimilatory reactions, remediatory roles, ecosystem, and regulatory functions of fungi has been discussed. The primary role of fungi is in the soil ecosystem**.** Additionally, this chapter provides information on commercial products derived from fungi that enhance soil health by way of nutrient acquisition, biocontrol, and bioremediation. The chapter also briefly describes the approaches through which fungi aid in restoring soil health.

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Keywords

Fungal products · Soil health · Assimilatory actions · Dissimilatory reactions · Remediatory roles · Ecosystem function · Regulatory function

3.1 Introduction

Soil is important for each and every living organism, and it denotes arrangement of sand, silt, clay, and other particles that are bound together with certain organic, inorganic, and chemical forces. Soil serves as a growth medium for plants and a source of nutrients and provides a habitat for a vast group of microorganisms, which convert insoluble materials into soluble materials that are easily absorbed by plant roots [1]. Soil health is crucial for crop management and maintaining a sustainable agricultural ecosystem. Health of soil is defined as its ability to function as a living ecosystem that sustains biological productivity, maintains air availability, and supports plant, animal, and human health [2]. There are countless microorganisms present in the soil, particularly where plant roots meet soil which is popularly known as the rhizosphere zone. Some microorganisms have neutral, beneficial, or fatal influences on plant development and survival, whereas others help their hosts through various processes [3].

Fungi are an important beneficial factor for agriculture due to their tremendous plasticity and survivability in adverse or unfavorable environmental conditions. Fungi are an important aspect of the soil ecosystem, as they decompose organic matter and recycle soil nutrients in making them available for plant growth. Table 3.1 summarizes the key role of fungi in the soil ecosystem. The table provides examples of fungi that are known to improve soil chemical (*Trichoderma harzianum* E15, *Glomus*), physical (*Rhizophagus intraradices*, *T. asperellum* ACCC30536), and biological (*Aspergillus flavipes*, *Trichoderma reesei*) properties. The fungi significantly contribute to increasing soil nutrient availability and soil biomass [4, 5]. The significance of fungal extracellular enzymes and their role in overcoming abiotic stress such as salt and drought are also depicted in Table 3.1.

3.1.1 Soil Health

Soil quality is primary in maintaining plant health, and plant health is directly linked to crop productivity. Soil is increasingly considered a nonrenewable resource because its recovery is a very slow process once it has been degraded [13]. Soil parameters describe the state of the soil ecosystem, in particular its production, buffering, filtration, and other processes. The structure of the soil profile (soil class), type, depth and skeletal nature, humus material and available nutrients, reactivity, foreign substances, and soil edaphon all appear to be critical. Physical, chemical, biological, and biochemical qualities sensitive to changes in the environment and land management have a substantial impact on soil quality. Soil parameters

Table 3.1 Impact of fungi on soil properties

S. no.	Fungi	Soil properties	Parameters studied	Impact on soil properties
1	*Glomus* and *Acaulospora*	Chemical	Relationships between AM fungi and soil P	Positive correlation between soil P and fungal spore count
2	*Trichoderma harzianum E15* and *Trichoderma virens ZT05*	Chemical	Rhizosphere soil nutrients	Rhizosphere soil nutrient content of *Trichoderma virens* ZT05 treatment was higher than *Trichoderma harzianum* E15 treatment
3	*Basidiomycota*, *Mucoromycota*, *Glomeromycota*, *Rozellomycota*, *Aphelidiomycota*, *Kickxellomycotina*, and *Planctomycetes*	Chemical	Bamboo biochar concentrations on fungal diversity and chemical composition	Positively affect nitrogen, phosphorus, carbon, and potassium
4	*Trichoderma asperellum CHF78*	Chemical	Nutrient uptake	Increased soil nutrient availability
5	*Septoglomus viscosum* and *Claroideoglomus claroideum*	Chemical	Soil degradation	Increased soil P and glomalin (a component of soil organic matter) content
6	*Trichoderma reesei*	Biological	Soil quality	Increase in soil respiration, and microbial biodiversity
7	*Suillus luteus*	Biological	Seedlings growth under different water conditions	Improved rhizosphere soil enzyme activity
8	*T. viride*	Biological	Soil microbial biomass	Increased microbial biomass of soil
9	*T. harzianum* T-22	Biological	Influence on rhizospheric microorganisms	Positive influence on microbial communities in the rhizosphere
10	*Aspergillus flavipes*	Biological	Soil enzymes	Soil enzymes (urease, acid phosphatase, invertase, and dehydrogenase) were significantly increased
11	*T. asperellum ACCC30536*	Physical	Soil moisture and pH	Positive effect on soil moisture and pH stability

(continued)

Table 3.1 (continued)

S. no.	Fungi	Soil properties	Parameters studied	Impact on soil properties
12	*Basidiomycota*, *Mucoromycota*, *Glomeromycota*, *Rozellomycota*, *Aphelidiomycota*, *Kickxellomycotina*, and *Planctomycetes*	Physical	Soil pH	Directly related to soil pH
13	*Trichoderma harzianum*	Physical	Soil salinity	Improved saline soil properties
14	*Rhizophagus intraradices*	Physical	Nutrient absorption, photosynthetic and antioxidant enzyme activities	Increased photosynthetic capacity, enhanced nutrient absorption, and increased antioxidant enzyme activities under salt stress
15	*Glomus lamellosum* and *Glomus etunicatum*	Environmental	Seedling growth under drought stress	AM fungi were effective means to improve the drought resistance of *C. migao* seedlings.
17	*Funneliformis mosseae BGC XJ01*	Environmental	Tobacco seedling growth in drought stress	Tobacco seedling growth significantly increased
18	*Rhizophagus irregularis* and *Funneliformis mosseae*	Environmental	Seedling growth under water stress	AMF can alleviate water-deficit damage and improve water stress tolerance
19	*Septoglomus deserticola* and *Septoglomus constrictum*	Environmental	Tomato plant tolerance under combined drought and heat stress	*S. constrictum* enhances the tolerance to drought and heat stress in tomatoes
20	*Funneliformis mosseae*	Environmental	Abscisic acid in *Zea mays* seedlings	Effective defense for better drought acclimation in water-stressed maize seedling
21	*Botryosphaeria rhodina*, *Trichoderma koningiopsis*, and *Neurospora* sp.	Agriculture management practices [3]	Screening of filamentous fungi under insecticide stress	Fungi are capable of degrading insecticide
22	*Aspergillus oryzae* and *Trichoderma longibrachiatum*	Agriculture management practices	Biodegradation of imidacloprid	Fungal isolates immobilized in sodium alginate and agar disc facilitated biodegradation of imidacloprid

(continued)

Table 3.1 (continued)

S. no.	Fungi	Soil properties	Parameters studied	Impact on soil properties
23	*A. fumigatus* and *A. niger*	Agriculture management practices	Biodegradation of chlorpyrifos	Fungi degrade pesticide (chlorpyrifos)
24	*Trichoderma harzianum* and *Metarhizium anisopliae*	Agriculture management practices	Organophosphate biodegradation	Fungi showed the ability to degrade organophosphorus insecticides
25	*Trichoderma viride*, *Penicillium funiculosum*, *Aspergillus flavus*, *Fusarium oxysporum*, *Rhizopus arrhizus*, and *Acremonium strictum*	Agriculture management practices	Chlorpyrifos biodegradation	Fungi degrade chlorpyrifos

Data sources: Jeffries et al., 2003 [6], Jimenez et al., 2016 [7], Li et al. 2019 [4], Mubekaphic et al., 2019 [8], Dellagi et al., 2020 [9], Li et al. 2021 [10], Tarin et al., 2021 [5], Wang et al., 2021 [11], Ali et al., 2021 [12]

are classified into physical, chemical, biological, and biochemical properties shown in Table 3.2. Physical properties involve bulk density, porosity, water retention capacity, soil temperature, etc. Chemical properties are total carbon and nitrogen content, soil reaction, and content of available nutrients. Biological properties are mainly microbial biomass and their activity, soil respiration rate and soil enzyme activity, etc. [14].

Physical properties such as soil texture, bulk density, depth, water-holding capacity, drainage, aeration, and soil structure are important in determining the acceptability of soil for agricultural, environmental, and technological purposes. Physical properties of the soil are associated with supporting capabilities, water and nutrient movement, retention, availability to plants, ease of root penetration, and heat and airflow [15]. Soil physical properties also influence soil chemical and biological properties. The physical properties of the soil have an important role in determining microbial abundance. The fungal community thrives in sandy soils, while the bacterial community thrives in clayey soils. The proportions and content of soil components have a significant impact on nutrient concentrations and soil texture, influencing the soil microorganisms [16]. Table 3.2 presents an ideal range of different physical, chemical, and biological soil parameters that are integral to supporting both plant and microbial biodiversity.

Soil chemical characteristics are linked to properties which have a direct impact on plant nutrition. Plants need several types of essential nutrients from the soil for their growth, and if any of the nutrients are inadequate in the soil, plants will not be able to grow properly. Chemical properties are soil pH, acidic, saline, sodic, and ion exchange phenomena (CEC and AEC). The most influential factor affecting soil

Table 3.2 Status of soil parameters and influence of fungi on soil properties

Soil parameters		Ideal range	Actual field status (Indian state mentioned in parenthesis)	Research on fungal contribution for the special soil parameters
Physical parameters	Critical bulk density	1.6–1.8 (gm/cm^3)	1.2–1.5 db (gm/cm^3) (district Kupwara, Kashmir Valley), 1.08–1.54 gm/cc (South Sikkim)	Norton et al., 2020 [20]; Fall et al., 2022 [21]
	Field capacity (FC)	0.17–0.32 vol/vol	42.2%, v/v (Katihar District in northeastern Bihar)	Norton et al., 2020 [20]
	Porosity (POR)	0.43–0.50 vol/vol	42 to 52,100 (db/dp*100) (District Kupwara, Kashmir Valley), 42.44 to 61.73% (South Sikkim)	Frąc et al., 2018 [22]; Lehmann et al., 2020 [23]; Norton et al., 2020 [20]
	Wilting point (WP)	0.025–0.20 vol/vol	16.5%, v/v (Katihar District in northeastern Bihar)	Norton et al., 2020 [20]; Fall et al., 2022 [21]
	Water-holding capacity	5–50%	54.18% (South Sikkim)	Frąc et al., 2018 [22]; Norton et al., 2020 [20]
	Moisture content	10–45%	0.68 to 4.32% (South Sikkim), 7.26–9.74% (Howrah districts, West Bengal), 3.92 (Delhi)	Frąc et al., 2018 [22]; Norton et al., 2020 [20]
Chemical	Organic carbon	0.5–7.5%	0.5 to 0.8% (district Kupwara, Kashmir Valley), 0.67% (Katihar District in northeastern Bihar)	Wilson et al., 2009 [24]; Zhu et al., 2016 [25]; Zhang et al., 2016 [26]; Mubekaphi et al., 2019 [8]
	Organic nitrogen	240–480 kg/ha	184 to 210 (kg ha^{-1}) (Buldana District, Maharashtra)	Zhu et al., 2016 [25]; Jansa et al., 2019 [27]
	Organic phosphorus	11–22 kg/ha	14 to 17 (kg ha^{-1}) (Buldana District, Maharashtra)	Xu et al., 2018 [28]; Zhang et al., 2018 [29]; Zhu et al., 2018 [30]; Rodrigues et al., 2021 [31]
	Organic potassium	110–280 kg/ha	130 (kg ha^{-1}) (Karnal)	Zhu et al., 2016 [25]; Choudhary et al., 2018 [32]; Kour et al., 2020 [33]

(continued)

Table 3.2 (continued)

Soil parameters		Ideal range	Actual field status (Indian state mentioned in parenthesis)	Research on fungal contribution for the special soil parameters
Biological/ microbial	Microbial biomass carbon	317.47 μg g^{-1}	0.5 to 0.8% (Buldana District, Maharashtra)	Albertsen et al., 2006 [34]; Welc et al., 2010 [35]; Zhu et al., 2016 [25]
	Soil respiration rate	1000–2000 mg CO_2-C/kg soil/ week	3.34 t C/ha/yr. (Howrah districts, West Bengal)	Gryndler et al., 2000 [36]; Albertsen et al., 2006 [34]; Verbruggen et al., 2012 [37]
	Soil temperature	50 to 75 °F	15–28 °C at soil surface (Vastrapur, Ahmedabad)	Albertsen et al., 2006 [34]; Hamel et al., 2017 [38]; Norton et al., 2020 [20]
	Cation exchange capacity	Calcium 65–80% of CEC, magnesium 10–15%, potassium 1–5%, sodium 0–1%, and aluminum 0%	5–30 (cmol (+)/kg) (North Coast soil)	Frąc et al., 2018 [22]; Norton et al., 2020 [20]; Fall et al., 2022 [21]
	Electrical conductivity	<1 milli mhos/cm^2	0.374–0.461 (dS m^{-1}) (Buldana District, Maharashtra), 0.37 (dS m^{-1}) (Karnal)	Choudhary et al., 2018 [32]; Norton et al., 2020 [20]; Fall et al., 2022 [21]
	pH	6.5–7.5	7.4–7.6 (Buldana District, Maharashtra), 8 (Karnal)	Ali et al., 2021 [12]; Norton et al., 2020 [20]; Jeffries et al., 2003 [6]

health is soil pH. Carbon availability, food availability, metal solubility, and biomass composition of fungi and bacteria are all influenced by soil pH [17]. Soil organic matter also affects soil health because this matter contains essential nutrients for soil health. Organic matter is critical for maintaining the nutritional quality and water-holding capacity of forest soils because it offers a suitable habitat and energy sources for the population of soil microbes [7].

The biological features of soil can impact the overall soil environment by influencing the concentration, availability, or decomposition of a specific element, which directly or indirectly affect the physical and chemical properties of the soil [18]. Soil biological properties are an important factor of soil health because without these plants may not survive in soil. The plant will not grow without the soil's

biological properties, even if the soil contains adequate physical properties and needed minerals or nutrients for their growth. Soil biological properties serve as a link between the soil and the plant that extracts nutrients from the soil by breaking down insoluble compounds into the soluble forms that plant roots can easily absorb. Several types of agrochemicals such as pesticides, fertilizers, and other types of synthetic chemicals are used in agriculture to improve crop production. Some of these compounds are slow in degradation and their residues remain in the soil for a long period. These chemical residues can affect soil health by affecting soil microbial population, soil chemical composition, and soil physical disturbance. Soil health is mainly affected by soil microorganisms which balance soil quality and fertility and influence soil health [19].

3.2 Role of Fungi in Soil Health Restoration

Dwindling arable land and depleting water and nutrient resources are stretching the limits of endurance of agricultural soils across the world. However, increasing population density is pressing demands for increasing agricultural productivity even at the cost of depleting soil quality and soil health. As a result, agricultural production must be greatly increased in the future decades in order to fulfill the massive food demands of the rising population. Excessive reliance on agrochemicals inevitably causes damaging effects on the environment [39]. Microbes are being recognized as an alternative to chemical fertilizers in agriculture due to their extensive capacity to increase crop production and food safety [40]. Biofertilizers can be used along with chemical fertilizers to offer a more cost-effective, environmentally sustainable solution. Various researchers have reported the effectiveness of biofertilizers in providing required nutrients to crops [41]. This section discusses the various mechanisms that biofertilizers employ to promote plant growth promotion and stress tolerance.

Plant growth-promoting fungi (PGPF) are rhizospheric fungi that colonize plant roots and promote plant growth. They produce phytohormones, organic acids, alcohols, antibiotics, siderophores, enzymes, and amino acids that make the environment conducive for plant growth either through direct or indirect mechanisms [42]. *Trichoderma*, *Penicillium*, *Fusarium*, *Piriformospora*, *Phoma*, and *Aspergillus* are some common PGPF [43]. These microbes can be found in a wide range of pH from acidic to alkaline. PGPF help plants either by enhancing their growth by secreting some phytohormones and other growth-promoting substances or by making the rhizospheric environment. Rhizospheric environment often contains phytopathogens and residues of toxic pesticides, heavy metals, and other xenobiotic compounds [44]. In ecological systems, fungi are the primary decomposers of substances as they feed on plant and animal tissues, both dead and living. It is widely known that fungi are exceptional decomposers of organic waste materials, such as cellulose, lignins, gums, and other organic complex substances. Additionally, fungi are crucial to mineral and water uptake, transferring and storing deficient nutrients to lackluster soil parts, and some other physiological processes [45]. These

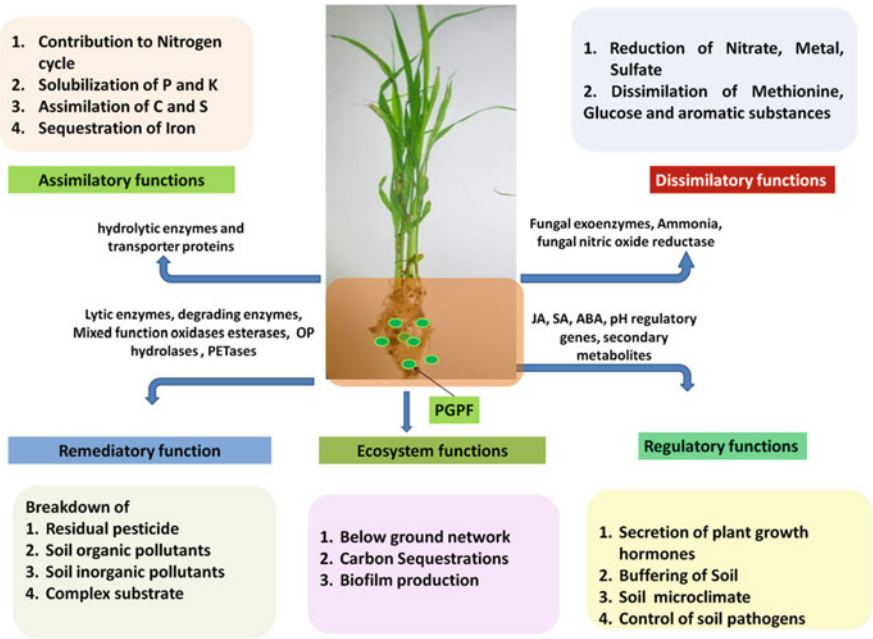

Fig. 3.1 Multiple functions of fungi in enhancing soil micro-ecosystem productivity

features of fungi can protect plants from various external stressors, such as pathogens, droughts, heat, salinity, cold, toxicity of pesticides and heavy metals, and so on [3]. By virtue of their functions that enhance soil micro-ecosystem productivity, fungi can be considered soil bioengineers. These functions can be categorized into five major categories: assimilatory, dissimilatory, regulatory, remediatory, and ecosystem functions (Fig. 3.1). However, all of these functions are interconnected and interdependent.

3.2.1 Assimilatory Functions

Assimilatory metabolism involves the incorporation of elements into the cellular structure [46]. Fungal assimilation is the process of absorption or acquisition of nutrients such as carbon, phosphorus, nitrogen, sulfur, and iron sequestration. In this manner, fungi help plants in acquiring nutrients from soil and hence play a role in their growth and development.

3.2.1.1 Contribution of Fungus to the Nitrogen Cycle

Nitrogen cycle in the soil and atmosphere is primarily a bacteria-driven scenario. Nitrogen (N) being a vital component for plant productivity and its reliance on actions on a few groups of microorganisms to bring into the soil environment is a

highly researched area in microbial biotechnology. Efforts to exploit the potential of beneficial microbes that can increase the symbiotic associations would significantly reduce the need of N fertilizers [9].

Biologically available nitrogen has marked intense research inputs during the earlier years of sustainable agriculture research. Multiple bacteria-based field studies and also a few products have emerged from this, for instance, *Rhizobium*-based products, e.g., Dr. Bacto's Rhizon, Utkarsh Rhizoz. As compared to bacteria, research on fungi for their contributions to the N_2 cycle is limited. Fungi especially help in assimilation, ammonification, and denitrification steps [47]. There is a limited information available on nitrogen fixation in fungi except for *Pleurotus* spp. In the soil, fungi and bacteria convert dead plants and animals into ammonia [48]. Some fungal genes, viz., *P450nor*, *Nirk*, and *dNar/aNar*, are reported to have a function in nitrogen cycling. Among the fungi, arbuscular mycorrhizae play an extremely important role in harvesting macro- and micronutrients [49]. The AMF can affect several biological processes, including biological N fixation, by increasing the growth of other microorganisms in the rhizosphere. By influencing the legume-*Rhizobium* symbiosis or by increasing the uptake of key nutrients like phosphorus, it also improves root nodulation and N_2 fixation [50]. Similar to this, endophytic fungi *Phomopsis liquidambaris* colonization remarkably escalated peanut productivity, nodulation, and N_2 fixation by secreting specific root exudates [51].

3.2.1.2 Phosphate Solubilization by Fungi

Several mechanisms have been observed by which phosphate-solubilizing microbes solubilize insoluble phosphates in soil, including the release of organic acids, phospholipases, alkaline phosphatases, and glucose dehydrogenases, as well as the excretion of compounds that can chelate ions and form complexes, thereby releasing phosphates for plant usage [52]. The main organic acids produced by fungal species *Trichoderma* and *Aspergillus* are lactic acid, fumaric acid, ascorbic acid, isocitric acid, malic acid, citric acid, phytic acid, glucuronic acid, gluconic acid, succinic acid, butyric acid, valeric acid, citric acid, fumaric acid, propionic acid, and acetic acid [53]. Organic acids attack phosphates and dissolve them, rendering the phosphorus usable for plants. The significance of mycorrhizal fungi both in forest ecosystems and the agriculture sector and organic farming has voluminous research that confirms their principal contribution toward P cycling. Members of both ectomycorrhiza (*Laccaria*, *Pisolithus*) and endomycorrhiza (*Glomus mosseae*, *Glomus aggregatum*, *Glomus etunicatum*, *Glomus deserticola*) have received the attention of researchers across the globe that led to products with proven yield benefits. These fungi are popularly known to produce a battery of extracellular enzymes including phosphatases (acids, alkaline) popularly associated with phosphate-soluble genes from fungi including pyrroloquinoline quinone (*pqq*) and glucose dehydrogenase (*gcd*). Phosphate-solubilizing fungi that may serve as potential biofertilizers are *Aspergillus niger*, *Penicillium*, *Trichoderma harzianum*, *Talaromyces*, *Rhizophagus irregularis*, *Glomus fasciculatum*, *Entrophospora colombiana*, and others [52]. *Aspergillus niger and Penicillium* sp. can potentially contribute to P nutrition of plants. *Aspergillus niger* is a key to unlocking fixed

phosphorus in highly weathered soils [10]. Wang et al. [54] demonstrated the capability of *P. oxalicum* y2 in solubilizing inorganic phosphorus as well as mineralizing organic phosphorus, which show their importance in utilizing as biofertilizers.

3.2.1.3 Potassium Solubilization by Fungi

Potassium-solubilizing fungi can be an invaluable resource for agriculture by enriching plants with potassium and enhancing crop quality [55]. Physicochemical forces, including pressure exerted by hyphae and root penetration, have also been demonstrated to enhance the desegregation of rock substrates by filamentous fungi. Through the secretion of protons, organic acids, and other compounds, fungi are involved in the biological weathering of rocks and minerals [56]. The four main methods that fungi employ to solubilize the mineral source of potassium are acidolysis, complexolysis, redoxolysis, and mycelial metal accumulation [57]. *Aspergillus* and *Penicillium* species are the most effective microbial inoculants for solubilizing potassium. *Aspergillus species* KSF 07, KSF 55, and KSF 69 solubilized 57–60 mg L^{-1} of K from mica and 34–39 mg L^{-1} from potassium aluminum silicate [58]. The ability of fungi to solubilize phosphorus, potassium sulfate, and iron as detailed below is the reason behind their potential for biomineralization.

3.2.1.4 Sulfur Assimilation by Fungi

The presence of sulfur is essential for plant growth and development due to its importance as a structural component of amino acids, vitamins, and cofactors [59]. It is also a constituent of signaling molecules that is involved in metabolic processes, stress tolerance, and complex signaling molecule cross talk. The primary source of sulfur in soil is SO_4^{2-} anion; it leaches out of the soil due to its high-water solubility. Sulfate is absorbed directly from the soil by plants via their sulfate transporters, as well as by microorganisms that live in symbiotic relationships with them.

Plants colonized with AMFs accumulate sulfur by accelerating its uptake from the soil, improving soil enzyme activities (extracellular sulfatases), or upregulating the expression of plant sulfur transporters. Considering AMF hyphae cover a considerably larger area than plant roots, and contain more sulfonate-desulfurizing bacteria as well as organo-sulfur-mobilizing microbes than bulk soil, they are an important site for microbial interactions for sulfur cycling. In low sulfur conditions, the endophytic fungus *Serendipita indica* enhanced sulfate uptake in maize plants [60]. An increase in sulfonate-mobilizing bacterial colonies was observed in AMF inoculated with *Lolium perenne*, increasing sulfur availability to the plant [61]. As fungi possess robust metabolic abilities, it is plausible that they could also contribute to the global sulfur cycle by mobilizing sulfur reserves in terrestrial ecosystems. Enzymes governing these metabolic activities in fungi are identified and characterized [62].

3.2.1.5 Iron Sequestration by Fungi

Cell growth and development are inextricably linked to iron (Fe). Iron is usually available as ferric ions, which are oxidized to form insoluble complex polymers containing oxyhydroxide under aerobic conditions. Under such conditions, the bioavailability of iron is significantly reduced. During reductive uptake, the external reduction of ferric salts, chelates, and proteins occurs prior to uptake. Siderophores, iron chelates, are selectively transported by these transporters, which participate in diverse intracellular interactions such as acidification of the external environment, reduction of ferric iron to the more soluble ferrous form, and secretion of soluble iron-chelating molecules [63].

A variety of microorganisms are capable of obtaining ferric iron from their extracellular medium, or external environment, via the formation of ferric iron-binding small molecules called siderophores [64]. Siderophores help in a localized concentration of iron particularly in the rhizosphere. Brown-rot fungi, *Candida*, and *Aspergillus* are reported to help in iron sequestration. Siderophores of fungi differ significantly in structure and are extremely diverse [65]. There are four main types of siderophores: hydroxamates, carboxylates, catecholates, and hydroximates among which the first two are predominant. Saprotrophic fungi *Aspergillus fumigatus* and *Aspergillus nidulans* synthesize more than 50 types of siderophores, especially under limited iron conditioning [64, 66]. To mobilize and distribute hyphal iron, *Aspergillus fumigatus* produces hydroxamate siderophores, triacetyl fusarine C, ferricrocin, and hydroxyferricrocin. Brown-rot fungi, however, may produce other types of catecholate siderophores besides ferricrocin (Frr) and ferrihordin. Fungi take up iron in a reductive and non-reductive manner.

3.2.2 Dissimilatory Functions

Dissimilatory metabolism occurs when elements are oxidized or reduced, and the organism uses the energy released during these processes [46]. Dissimilatory nitrate reduction and formation of ammonium play an important role in the terrestrial nitrogen cycle [67]. *Fusarium oxysporum* and *Cylindrocarpon tonkinense* are well-known fungi that could reduce nitrate in a dissimilatory manner. Apart from these, the vast bulk of fungus found on land, including ectomycorrhizal fungi, exhibited this trait. Since fungal denitrification doesn't include a reduction step, fungi are very important to soil nitrous oxide (N_2O) producers. In fungi, ambient oxygen levels influence two routes for dissimilatory nitrate reduction. Under hypoxic conditions, denitrification takes place and under anoxic levels, ammonia fermentation takes place [68]. Key genes involved in fungal denitrification are NirK and P450nor. NirK codes for copper-containing nitrite reductase which reduces nitrite to nitric oxide (NO). The cytochrome P450 nitric oxide reductase (P450nor) catalyzes the conversion of NO to N_2O. Fungi lack nitrous oxide reductases, which is why the final product of fungal denitrification is N_2O rather than N_2. In ammonia fermentation, NiaD (nitrate reductase) and NiiA (nitrite reductase) convert nitrate to ammonium. These processes could significantly contribute to the emission of N_2O

from soils and the removal of nitrogen from nitrate- and nitrite-contaminated environments [69].

Soil fungi can dissimilate methionine in the presence of glucose. When glucose is present, all of the sulfur (from methionine) is released as methanethiol, with a part of it being oxidized to dimethyl disulfide. Other carbon residues were α-hydroxybutyric acid and α-aminobutyric acid. The fungus' inability to metabolize α-keto butyrate resulted in its inability to use methionine as a carbon and energy source. Several additional soil-isolated fungi flourished on α-amino butyrate but not on methionine due to their inability to demethiolate it [70]. Dissimilation and catabolism of aromatic substances by fungi are elaborated under remediatory function in detail.

3.2.3 Remediatory Function

Soil is made up of various essential components such as organic matter and soil water and air of earth that directly or indirectly sustains life. Soil presents 15 huge varieties of nutrients (carbon, oxygen, hydrogen, nitrogen, phosphorus, potassium, sulfur, magnesium, calcium, iron, boron, manganese, zinc, molybdenum, copper) and wide range of trace elements, inorganic compounds, and organic compounds which are required for growth and development of plants. Soil factors such as pH, calcium carbonate (CaCO3), (organic matter) humus, and cation/anion exchange capacity, as well as the soil microenvironment, all have an impact on the accessible soil micronutrients. Table 3.2 shows summarized information regarding different soil parameters and ideal range of physical biochemical and biological parameters. Table 3.2 also depicts present status with respect to specific parameters found in certain Indian states. A fertile soil preserves the water quality and air quality, allowing species to thrive and resisting pressures such as anthropogenic activities, atmospheric conditions, and environmental deterioration as well as promoting nutrient delivery, crop productivity, and individual health [71].

Soil pollution is defined as the assemblage of persistent toxic chemical compounds, elements, salts, explosives, metals, metalloids, radioactive elements, and infectious agents that all show deleterious effects on living organisms, mainly plants and animals. Agriculture requires productive soils in order to feed the globe adequately [72]. Soil pollutants are divided into two categories: organic pollutants and inorganic pollutants, both of which when in excess are responsible for deterioration of soil quality. Leaching and irrigation/flooding runoff introduce these pollutants in soil to underground aquifers and surface water bodies. Because of their poisonous nature and the anomalies they cause, their presence is undesirable in soil and water resources. Seed germination, plant growth, soil microbial activity, and the ability of plants and microorganisms to absorb water and nutrients from the soil are all harmed by the presence of hydrocarbons and metals and other pollutants in soils. As a result, removing these hazardous compounds from the soil is one of the top priorities in environmental research [73].

3.2.3.1 Organic Pollutants

Organic compounds created naturally or anthropogenically tend to accumulate in the soil. Soil pollution is a severe environmental issue that has a global impact. Exposure to contaminated soils has been linked to a variety of negative health impacts, according to a significant body of data; soil pollution is caused by massive amounts of organic chemicals used in farming and metropolitan regions. Anthropogenic activities, industrial activities, combustion of coil, automobile emissions, waste disposal, and sanitary landfills are also the main causes of soil pollution. Apart from domestic, industry generated soil pollution; farmers have been employing a growing number of organic compounds in agricultural areas in order to offer acceptable amounts of agricultural products, which in turn are resulting in huge amounts of pollution and environmental degradation. Soil consists of various organic pollutants such as pesticides (organophosphates, carbamates, endosulfan), herbicides, organic fuels, PCBs, PBBs, PCDs, PAHs, and insecticides, particularly nonrenewable fossil fuel (gasoline and diesel) [74, 75].

Fungal cell biomasses metabolize/decolorize azo dyes by absorbing dyes and liberating extracellular enzymes. Fungal enzymes are also vital in the degradation of the hazardous by-products produced by azo dyes. Fungal enzymes mainly such as laccase, lignin peroxides, and manganese peroxides aid in decolorization of azo dyes [76]. Peroxidases are classified into three types based on their source and activity: lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP) among the peroxidase-catalase superfamily.

When MnP, LiP, and VP are produced by the same organism, they might work together to oxidize lignin-like structures. White-rot and basidiomycetous fungi are commonly used for the degradation of hazardous substances by heme peroxidase enzymes (LiP and MnP) in the presence of manganese and hydrogen peroxide. On the flip side, VP enzymes are invaluable in bioremediation processes, and they are also substrate-specific enzymes that can oxidize both phenolic and non-phenolic substances. Some other heme peroxidases like dye-decolorizing peroxidases (DyPs) and unspecific peroxygenase (UPO) catalyze the oxidation of non-phenolic lignin compounds and other organic compounds in the presence of hydrogen peroxide. A peroxidase generated by *B. adusta* has been demonstrated to break the phthalocyaninic ring in phthalocyanine dyes by cleaving the azo link, resulting in azo and phthalocyanine dye decolorization. Laccase, being the most frequent member of the multicopper oxidase protein family, shows significant promise as a biodegradation enzyme. Laccase uses molecular oxygen to oxidize aromatic and nonaromatic compounds while abstracting protons and generating radicals during the process. Degradation by laccases is considered a unique bioremediation process because of its nonspecific oxidation capacity, no essentiality of cofactors and oxygen as an electron acceptor [77, 78].

3.2.3.2 Pesticide Biodegradation

White-rot fungus has the potential to bioremediate organic chemical compounds that are difficult for bacteria to degrade such as herbicides, insecticides, pesticides, and weedicides. Fungi's ability to release extracellular enzymes that interact with a wide

range of chemical compounds gives them the potential to remediate organic contaminants [79]. The degradation of pesticides by fungus can be separated into three phases: (a) during the initial phase, the parent pesticidal compound is reduced, hydrolyzed, or oxidized into nontoxic and soluble compounds. Reductive and oxidative enzymes released by fungi play a critical role in this process. (b) During the next phase which is conjugation, the water solubility of the intermediate compounds is enhanced. (c) During the final phase of the process, peroxidases, oxygenases, and other enzymes transform intermediary molecules into nontoxic molecules [80].

Esterases, glutathione S-transferases (GSTs), and cytochrome P450 are the three primary enzyme groups involved in the degradation of complex organic molecules [79]. Involvement of laccase in the breakdown of pesticides dicofol and chlorpyrifos was deduced from a study by Hu et al. (2020) [81]. In this study, laccase produced by the fungus *Trametes versicolor* was explored. Dechlorination is the first step in the process, which produces 2,2-dichloro-1,1-bis-(4-chlorophenyl)-ethanol from dicofol. In the next step, oxidative cleavage and dechlorination produce 4,4′--dichlorobenzophenone. In the final step, lignin-degrading enzymes split the molecule's ring structure, releasing benzaldehyde [81]. In the breakdown of xenobiotics, cytochrome p450 enzymes have been widely researched. Despite the fact that the enzymes that have been purified directly were not used in the process, certain outcomes from the use of cytochrome p450 inhibitors suggested that those enzymes are critical in the degradation mechanism. Major intermediates generated by the demethylation of diuron are 1-(3,4-dichlorophenyl)-3-methyl urea (DCPMU) and (3,4-dichlorophenyl) urea. Of these three, only DCPMU is amenable for further breakdown by fungal enzymes. Diuron hydrolysis by cytochrome p450 and laccases was seen in basidiomycete strains. By acting as monooxygenases, heme-thiolate proteins aid in the hydroxylation, dehalogenation, deamination, and dealkylation of pesticides. Under circumstances where laccases aren't engaged in the reaction, intracellular cytochrome p450 was found to act as a catalyst [82].

3.2.3.3 Plastic Biodegradation

Plastic depolymerization by microorganisms and/or enzymes is a potential approach for degradation of petro-plastic wastes into simple monomers. These monomers can be recycled or processed to yield CO_2 and H_2O, as well as valuable by-products. Various extracellular enzymes have the capability to interact with the surface of the plastic and break it down into short intermediate polymers. Short polymers are also digested by microbial cells (carbon source), resulting in the production of CO_2. On the basis of various bonds in polymeric chain, the biodegradation mechanism of petro-plastic can be categorized into three classes: (a) carbon back-bones polymers, (b) ester-bond back-bones and side-chains polymers, and (c) polymers with hetero−/carbamate(urethane) bonds [83].

Enzymes such as lignin peroxidases, hydrolases, imprecise peroxygenase, laccases, manganese peroxidases, and depolymerase aid in the decomposition of plastic substances. In the process of enzyme-based degradation, depolymerase enzymes fragment the ester linkages of polyethylene terephthalate (PET) which

further proceed for complete breakdown. Polyurethane-degrading fungi *Cladosporium* sp., *Penicillium chrysogenum*, and *Aspergillus fumigatus* were shown profuse esterase activity in comparison to protease and urease activities. Serine hydrolase has been found to be responsible for breakdown of polyurethane by *Pestalotiopsis microspora*. Fenton's technique is popularly used in the depolymerization of polystyrene sulfonate (PSS) by *Gloeophyllum trabeum* DSM 1398. Additional processes involving hydroxyl radicals in the oxidation pathway include aromatic ring substitution and the generation of tertiary radicals. Polyethylene-digesting enzymes help to degrade polyethylene (PE) into mono(2-hydroxyethyl) terephthalic acid (MHET). The hydrolysis of MHET to ethylene glycol and terephthalic acid is then completed by the MHET-digesting enzyme [84, 85].

3.2.3.4 Inorganic Contaminants

Inorganic pollutants are made by dissolving forms of cations and anions of various toxic metals, nutrients, and salts. Potentially toxic elements (PTEs) are compounds that exist widely in all human environments. These compounds usually arise after partial degradation of parent material. In recent years, potentially hazardous materials are increasingly encountered specifically in soil and sediment ecosystems [86]. Potential toxic elements are found in large quantities in soil (PTEs). PTE concentrations and availability can have an impact on the soil ecosystem as well as plant growth and development. PTEs can enter into the human body either directly (via inhalation or ingestion of soil particles) or indirectly (through plants) resulting in catastrophic health consequences. In many deteriorated soils, metal pollution is a recurring problem. Cadmium (Cd), nickel (Ni), copper (Cu), chromium (Cr), lead (Pb), zinc (Zn), mercury (Hg), and arsenic (As) are the most frequent metals (Hg). As and Cd are exceedingly poisonous, while Pb, Ni, and Hg are somewhat poisonous, and Zn, Cu, and Mn are considerably less harmful [87]. Under the natural process, microorganisms (fungi, bacteria) and plants assist to eliminate or degrade the toxic or harmful substances present in soil and water, thereby preserving a clean or nontoxic environment called bioremediation. The term "remediation" refers to the process of removing a problem, and "bioremediation" refers to the process of removing an ecological problem such as soil and groundwater contamination. Bioremediation is a process that employs the use of live microorganisms to mitigate or prevent natural pollution. It's a progression toward removing poisons from the environment, restoring the environment's original characteristics, and preventing new contamination. Various types of remediation techniques are used to remove heavy metals, metalloids, and other inorganic contaminants from soil or water such as bacterial bioremediation, phycoremediation, phytoremediation, and mycoremediation [88].

3.2.3.5 Mycoremediation

Fungi appear to be effective bioremediation agents in detoxifying organic contaminants and removal of heavy metals. Fungi have a high adsorption and accumulation capability for HMs; therefore, they could be useful. Bioaccumulation, bioadsorption, biosynthesis, biomineralization, bioreduction, bio-oxidation, extracellular precipitation, intracellular precipitation, surface sorption, and other

biomechanisms play a role in HM tolerance and fungal eradication. The efficiency of mycoremediation varies from species to species. Various fungal strains and their enzymes used in remediation of organic and inorganic pollutants such as pesticides, plastics, azo dyes, persistent organic compounds, and heavy metals are presented in Table 3.3. Major influential parameters that affect the bioremediation of HMs are time, pH, temperature, HM concentration, a dose of fungal biomass, and rate of shaking/stirring that vary according to the fungi and various types of heavy metals [89]. Khan et al. (2019) [90] identified indigenous fungal strains having the capacity of soil remediation of heavy metals. *Penicillium rubens* (M2Aii), *Aspergillus fumigatus* (M3Ai), and *Aspergillus niger* (M1DGR) were the main three metal-tolerant fungi. Among them *A. niger* was shown to be a more suitable fungus for removing hazardous heavy metals (Cd and Cr) from polluted soil.

3.2.3.6 Methods for Mycoremediation

Biosorption is a key process in environmental protection as well. The physicochemical interactions between the metal and the functional groups present at the surface of the biosorbent cause heavy metal and organic compound biosorption. Physical adsorption, ion exchange, and chemical sorption are among the mechanisms involved, which are unrelated to metabolism. Microorganisms' cell walls are mostly polysaccharides, proteins, and lipids, with carboxyl, sulfate, phosphate, and amino groups to create interactions with metals and their complexes. This type of biosorption happens quickly and can be reversed. The structure of organic contaminants varies substantially. As a result, molecule size, charge, solubility, hydrophobicity, and reactivity all affect biosorption [91].

Laccases, proteases, lipases, cellulases, catalases, amylases, xylanases, and peroxidases, among others, are industrially important enzymes that can be utilized to degrade organic pollutants from the environment. These enzymes also aid to decompose polymeric compounds like protein, xylan, lipids, cellulose, starch, and other polymeric molecules found in agri-food industry and other domestic waste effluents. Then the digested end products can be deteriorated to produce valuable chemicals like fatty acids and biogas. White-rot fungi produce a ligninolytic enzyme that not only is used to degrade natural form of lignin but also aids to decolorize the organic contaminants like natural and synthetic dyes which are commonly found in environment. Peroxidases and laccases are the two types of extracellular ligninolytic enzymes responsible for lignin oxidation. In oxido-reductive catalyzing processes, peroxidases use hydrogen peroxide as final electron acceptors, while laccases and monooxygenases use molecular oxygen as final electron acceptor. Due to their decreased substrate specificity, there is an evidence indicating the importance of enzymes in the bioremediation and decontamination of harmful organic and inorganic contaminants [92].

The mechanism of mycoremediation is figuratively represented (Fig. 3.2) where different types of organic and inorganic compounds are categorized into plastics, pesticides, and automobile and pharmaceutical wastes. These polluting substances leach out harmful intermediates. Remediation of these substances principally involves biosorption and enzymatic degradation. These methods are cheap,

Table 3.3 Role of fungi in bioremediation of environmental pollutants

Category	Types of pollutants	Enzymes	Name of fungus	References
Pesticides	Organochlorines (endosulfan)	Phenol hydroxylase	*Trichosporon cutaneum*	Singh et al., 2019 [93]
	Organophosphates (profenofos, monocrotophos)	Phosphotriesterases, alkaline phosphatases, esterases, OP hydrolases	Marine fungi (*A. sydowii* CBMAI 935 and *P. raistrickii* CBMAI 931), *Trichoderma harzianum* T103	Da Silva et al., 2013 [94] Kumari and Sundari, 2022 [40]
	Atrazine (2-chloro-4-ethylamino-6-isopropylamino-S-triazine; AT) and alachlor (2-chloro-, −diethyl-N-[methoxymethyl]-acetanilide)	Extracellular (ligninolytic enzymes)	White-rot fungus, *Phanerochaete chrysosporium*	William et al., 2011 [95]; Henn et al., 2020 [82]
	Diuron	Cytochrome p450 and laccases	Basidiomycete strains	Henn et al., 2020 [82]
	Dicofol and chlorpyrifos	Laccases	*Trametes versicolor*	Huang et al., 2018 [80]
POPs	PCDDs (polychlorinated dibenzo-p-dioxins)	Cytosolic and mitochondrial isoforms of P450 monooxygenases	*Fusarium oxysporum*	Sakaki et al., 2013 [96]
	PCBs (polychlorinated biphenyls)	Intracellular (cytochrome P450 monooxygenase, aryl-alcohol dehydrogenase, and aryl-aldehyde dehydrogenase) and extracellular (ligninolytic enzymes)	Ligninolytic fungal strains *Irpex lacteus* 617/93, *Bjerkandera adusta* 606/93, *Phanerochaete chrysosporium*	Čvančarová et al., 2012 [97]
	PAHs (polyaromatic hydrocarbons)	Laccase, manganese peroxidase enzyme	White-rot fungi such as *T. versicolor*, *P. ostreatus*, *Irpex lacteus*	Rosales et al., 2013 [98]; Kumari et al., 2021 [92]
Azo dyes	Brilliant blue	MnP (manganese peroxidase) and Lac (laccase)	*Phanerochaete chrysosporium* and *I. lacteus*	Pazarlioglu et al., 2010 [99]; Singh et al., 2020 [76]
	Indigo carmine	Laccase	White-rot fungus, *T. hirsute*	Couto, 2006 [100]

(continued)

Table 3.3 (continued)

Category	Types of pollutants	Enzymes	Name of fungus	References
	Blue dye	MnP (manganese peroxidase)	*P. chrysosporium*	Pazarlioglu et al., 2010 [99]
	Acid red 97	Laccase	*Peroneutypa scoparia*	Pandi et al., 2019 [101]
	Scarlet RR	LiP (lignin peroxidase), laccase, and MnP (manganese peroxidase)	*Peyronellaea prosopidis*	Bankole et al., 2018 [102]
	Acid red 3	MnP (manganese peroxidase)	*Trichoderma tomentosum*	He et al., 2018 [103]
Heavy metals	Lead (pb II)	Anti- oxidative enzymes including catalase	*Aspergillus foetidus*	Chakraborty et al., 2013 [104]
	Mercury	Biosorption	*Candida parapsilosis*	Muneer et al., 2013 [105]
	Chromium	Biosorption	*Aspergillus versicolor*, *Gloeophyllum sepiarium*, *Saccharomyces cerevisiae* (Y)	Parvathi et al., 2007 [106]
	Nickel	Biosorption	*Aspergillus* sp., *Aspergillus versicolor*, *Aspergillus niger*	Taştan et al., 2010 [107]
	Copper (Cu II)	Copper-amine oxidase enzymes	*Aspergillus* spp., *Aspergillus versicolor*, *Aspergillus niger*	Mitra et al. 2014 [108]
Plastics	Polyurethane	Serine hydrolase	*Pestalotiopsis microspora*	Russell et al., 2011 [109]; Brunner et al., 2018 [110]
	Polyethylene	PHB-depolymerase (3-poly hydroxybutyrate), laccase, and manganese peroxidase	*Penicillium pinophilum*, *Penicillium simplicissimum*	Ojha et al., 2017 [111]
	PHB, PBS, PES, PCL	Polyhydroxybutyrate depolymerase, bifunctional lipase-cutinase	*Aspergillus fumigatus*	Jung et al., 2018 [112]; Zuo et al., 2019 [113]; Kaushal et al., 2021 [114]

(continued)

Table 3.3 (continued)

Category	Types of pollutants	Enzymes	Name of fungus	References
	PBS, PLA	Proteinase K and lipase	*Candida rugosa/ Tritirachium album*	Hu el al., 2018 [115]; Hegyesi et al., 2019 [116]; Kaushal et al., 2021 [114]

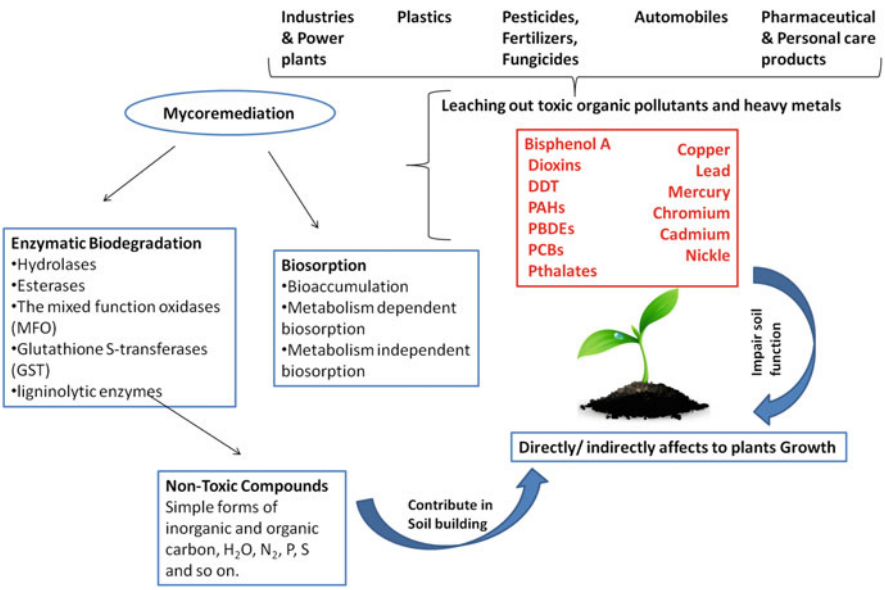

Fig. 3.2 Mechanism of mycoremediation

significant, and commonly used for remediation of toxic pollutants into nontoxic compounds. In the enzyme degradation method, various enzymes are involved such as hydrolases, esterases, the mixed function oxidases, glutathione S-transferases and ligninolytic enzymes in the biodegradation of organic and inorganic pollutants. These enzymes are mainly produced by fungal strains like *Phanerochaete chrysosporium*, white-rot fungal strains, and *Irpex lacteus* and others; their function is represented in Table 3.3. Biosorption of heavy metals follows different types of mechanisms such as bioaccumulation, metabolism-dependent biosorption, and metabolism-independent biosorption. At the end of the breakdown process, nontoxic end products will directly and indirectly contribute to soil building and plant growth.

3.2.4 Ecosystem Function

Fungi can be found in abundance in natural ecosystem soils and play an important part in ecosystem functioning. Intensively managed soils have low fungal biomass which adversely impacts soil functioning. As a result, fungal sequestration of carbon depends on the presence of fungal activity, which captures and transports a myriad of organic substances from root zones to deeper soil layers, alongside transporting these organic substances into aggregation zones where organic matter binds with minerals [117]. Fungi use carbon to construct networks in the soil and create nutrition webs when they adhere to plant roots. They have mutual association with plants, for instance, they give phosphorus to plants in exchange of carbon dioxide.

Saprotrophic fungi that perform a scavenging role and also produce hyphae can contribute to carbon stability through carbohydrate component translocation in the soil matrix, which collects organic material with soil particles and occludes OM in aggregates [118]. By virtue of producing a wide variety of extracellular enzymes, fungi break down a variety of organic matters and hence regulate the balance of carbon and other nutrients [22]. It has been found that applying fungal biomass at the start of the growing season improves crop productivity by boosting overall soil health. Increased fungal activity leads to greater aggregate formation due to fungal enmeshment, which results in carbon being trapped inside aggregates and therefore being physically protected from further degradation [119].

In addition to mutualistic association, some fungi also have facultative/obligatory symbiotic association with terrestrial plants. AM fungi are in symbiotic association (facultative) with the majority of agricultural and horticultural plants. EM harbor tree species principally. Orchid and ericoid mycorrhizae border on obligate symbiosis. However, certain host functions failed to manifest in the absence of fungal symbiosis. There is a lot of evidence that ecosystems with a healthy mycorrhizal fungi network can store eight times more carbon in the soil than those without. This suggests that fungi-friendly agroecosystems have a significant potential for storing more carbon in the soil.

3.2.5 Regulatory Function

Fungi regulate the various functions in the soil such as nutrient cycling, water dynamics, and suppression of harmful microbes. They are the main decomposers in the soil food webs and share the responsibility to convert complex indigestible organic material into available forms. Fungal hyphae bind soil particles together to build aggregates, which improve water infiltration and soil water-holding capacity. They exhibit high plasticity and adapt in response to adverse or unfavorable conditions. By changing the soil microclimate, soil fungi help plants to tolerate various environmental stresses such as drought, salinity, metal, etc. Biological (plants and other organisms) and abiotic (soil pH, moisture, salinity, structure, and temperature) factors influence the diversity and activity of fungi. There are fungi in almost every habitat and they can survive in a wide range of pH and temperature

[120]. Furthermore, arbuscular mycorrhizal symbiosis is a mutualistic relationship that occurs between most terrestrial plants and fungi. Numerous signaling processes between and within the symbiotic partners are involved in the beginning, development, and control of this symbiosis. Phytohormones, among other signals, are recognized to play an essential role at various phases of the relationship. Plant growth-promoting fungi (PGPFs) are the heterogeneous group of nonpathogenic fungi that can be obtained in the rhizosphere, at the root surfaces, or inside the roots of plants. The PGPFs not only provide the plant with the root extension, plant growth development (seed germination and seedling vigor and photosynthetic efficiency), and protection from various kinds of phytopathogens but also help in the soil improvements. Auxins, cytokinins (CKs), gibberellic acids (GAs), ethylene (ET), abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) are also produced by fungi. In response to biotic and abiotic stress, these hormones play a key role in controlling plant development [121]. Fungi have a crucial role not only in plant growth, and soil health, but also in plant disease control. Fungi such as *Trichoderma harzianum*, *T. viride*, *T. gamsii*, *Metarhizium anisopliae*, *Acremonium alternatum*, *Coniothyrium minitans*, *Ampelomyces quisqualis*, *Aspergillus flavus*, and *Chondrostereum purpureum* and others are used in the biocontrol of pathogens and also used in bioformulation production. For instance, *Trichoderma* is a genus renowned for its biocontrol activity against several pathogenic species, and it is used against the control of a variety of plant diseases, including onion white rot, chickpea wilt, and tomato crown and root rot [122]. *Metarhizium anisopliae* is a hyphomycete entomopathogenic fungus that is found all over the world and is used to control insects. A moist soil is the ideal environment for filamentous growth and for the production of infectious spores known as conidia that infect soil-dwelling insects [123]. Fungi are being used as a biocontrol in several countries throughout the world. Several species of fungi have been used in the development of fungicides in recent years such as Biokuprum™ (from *Ch. cupreum*), Promote® (from *Trichoderma harzianum*, *T. viride*), Trichodex® (from *Trichoderma harzianum*), SoilGard® (from *Gliocladium virens*), and Ketomium® (from *Chaetomium globosum*) [124].

The application of fungi as biocontrol agents is beneficial due to their target-specific nature, ability to remain in the soil for prolonged periods of time without requiring repeated inoculation, and environmentally friendly nature. Fungi use four types of mechanisms to control pathogens, namely, hyperparasitism, antibiosis, competition, and synergism.

In hyperparasitism, a biocontrol agent (BCA) attacks the pathogen directly, killing it or its propagules. Several fungi such as *Acremonium alternatum*, *Acrodontium crateriforme*, *Ampelomyces quisqualis*, *Cladosporium oxysporum*, and *Gliocladium virens* control diseases such as powdery mildew by using the hyperparasitism mechanism (Milgroom and Cortesi, 2004). Soil fungi attack plant pathogenic fungi as mycoparasites and inhibit their growth or kill them. *To detect pathogens*, *Trichoderma* produces extracellular exochitinase, which catalyzes the dispersal of cell wall oligomers from the pathogens [11].

In antibiosis, fungi produce antibiotics, volatile compounds, and metabolites as represented in Table 3.4. *Trichoderma* produces several types of secondary

Table 3.4 Metabolites produced by fungi against pathogens

S. no.	Fungi	Class	Metabolites	Pathogen	Biocontrol activity
1	*Chaetomium globosum* F0142	Sordariomycetes	Chaetoviridins A and B	*Magnaporthe grisea* and *Puccinia recondita*	Antifungal
2	*Trichoderma harzianum* T22 and T39		Azaphilone, butenolide, 1-hydroxy-3-methyl-anthraquinone, 1,8-dihydroxy-3-methyl-anthraquinone, harzianolide, and harzianopyridone	*Rhizoctonia solani*, *Pythium ultimum*, and *Gaeumannomyces graminis*	Antifungal
3	*Pythium oligandrum*	Oomycetes	Tryptamine and oligandrin	*Pythium dissotocum*	Antifungal
4	*Talaromyces wortmannii*	Eurotiomycetes	Wortmannin, emodin, wortmannin-diol, wortmin, emodic acid, skyrin, oxyskyrin, rugulosin A, and rugulosin B	Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Enterobacter* sp., and *Enterococcus cloacae*) and Gram-positive bacteria (Methicillin-resistant *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumonia*, and *Enterococcus faecalis*)	Antibacterial
5	*Trichoderma harzianum-SQR*, *T037*	Sordariomycetes	Harzianolide	*Sclerotinia sclerotiorum*	Antifungal
6	*Trichoderma pseudokoningii* SMF2	Sordariomycetes	Trichokonins	*Pectobacterium carotovorum*	Antibacterial
7	*Penicillium chrysogenum* V11	Eurotiomycetes	Penochalasin K, chaetoglobosin C, and penochalasin I	*Colletotrichum gloeosporioides* and *Rhizoctonia solani*	Antifungal
8	*Aspergillus niger*		Oleic acid, n-hexadecanoic acid, hexose, glycerol, stearic acid, tetradecanoic acid, dodecanoic acid, and 5-hydrxoymethylfurfural	*Pyricularia oryzae*	Antifungal

(continued)

Table 3.4 (continued)

S. no.	Fungi	Class	Metabolites	Pathogen	Biocontrol activity
9	*Aspergillus tritici SP2–8-1*	Eurotiomycetes	4-methyl-candidusin A, aspetritone A, aspetritone B, prenylcandidusin derivatives, candidusin derivatives, terphenyllin derivatives, and anthraquinone derivatives	*Staphylococcus aureus*, *Vibrio vulnificus*, *vibrio rotiferianus*, *Vibrio campbellii*, and significantly stronger cytotoxic activities against human cancer cell lines *HeLa*, *A549*, and *Hep G2* than the other compounds	Antibacterial
10	*Aspergillus candidus SFC20200425-M11* *Aspergillus montenegroi SFC20200425-M27*	Eurotiomycetes	(R)-formosusin A, (R)-variotin, candidusin, asperlin, montenegrol, and protulactone A	Fungi (*Alternaria brassicicola*, *Botrytis cinerea*, *Colletotrichum coccodes*, *Fusarium oxysporum*, *Magnaporthe oryzae*, *Phytophthora infestans*) and bacteria (*agrobacterium tumefaciens*, *Clavibacter michiganensis*, *Pseudomonas syringae*, *Ralstonia solanacearum*, *Erwinia amylovora*)	Antifungal and antibacterial

Data sources: Cai et al., 2013 [125], Li et al., 2021 [10]

metabolites such as koninginins, viridins, azaphilones, and diketopiperazines which are important in biocontrol of pathogens (Sood et al., 2020). It has been long recognized that microbes, particularly fungi, offer an abundant source of novel metabolites. There are about 23,000 identified important microbial secondary metabolites with fungi producing 42% of them [126]. Fungal secondary metabolites are known to be involved in signal transduction and catalysis. Salicylic acid, acetylsalicylic acid, and nitric oxide have properties that boost host plant immunity and resistance [127]. In addition to controlling pathogens, some of these compounds also appear to increase plant vigor, possibly by increasing hormone synthesis. For instance, harzianolide, produced by *Trichoderma* species, may assist both defense and growth responses [125]. By releasing volatile and nonvolatile antibiotics, *T. harzianum* and *T. viride* were seen to be particularly effective in reducing the radial growth of *S. rolfsii*. Another mechanism of biocontrol is competition. Soil nutrients are important for every living organism. These soil nutrients are required for their growth and development. Fungi compete with pathogens for nutrients and space and suppress pathogens by rapidly proliferating. An example of biocontrol through competition is between *Trichoderma* sp. and *Fusarium* sp. [128]. *Trichoderma* species have a high capacity for mobilizing and utilizing soil resources, making them more efficient and competitive than other soil microorganisms. *Trichoderma* species also produce siderophores, an iron-chelating compound that not only serves as an iron-chelating compound but also suppresses phytopathogen growth by depriving them of iron supplies. There are a good number of examples where fungi form synergistic associations with bacteria (referred to as helper bacteria) delivering compounded benefits to the host plants.

Some fungi have the ability to combine hydrolytic enzymes and antibiotic secondary metabolites in their activities. An example of synergism is AM fungi either alone or in combination with bacteria help in plant growth and development [129]. *Trichoderma* is used as a biocontrol agent against pathogens and it is also helpful in plant growth and development as a result of the synergistic effects of antimicrobial compounds. For example, *Trichoderma* in association with *Pseudomonas* was reported to produce cell wall-degrading enzymes and membrane-disrupting lipo-depsipeptides and control fungal pathogens [130].

3.3 Status of Commercial Microbial Inputs

Recognizing detrimental effects of agrochemicals (fertilizers, pesticides, and other synthetic chemicals) in agriculture, scientists are making a determined effort to move toward an alternative to these agrochemicals. There are thousands of microorganisms in soil which are used for plant growth and development as well as protection from pathogens. Beneficial microorganisms such as mycorrhizal fungi, plant growth-promoting rhizobacteria (PGPR), and root endophytic fungi are important and their ability to produce regulatory and growth promoter compounds such as phytohormones, and secondary metabolites, and play an important role in nutrient processes including nitrogen cycling, carbon cycling, and phosphate solubilization.

Due to current public awareness about agrochemicals and their negative impacts, there is a growing interest in learning more about microbial contribution to safe agriculture. Microorganisms can be used as a low-input biotechnology where fungal species can be variously employed to promote sustainable, environmentally friendly activities that are critical to the stability and productivity of both agricultural and natural ecosystems [131]. Scientists and researchers have been working on the isolation and identification of plant-beneficial microorganisms from past years, but only a few have made it to the commercial market because many bio-inoculants do not perform as well in the field as they perform in greenhouse or laboratory tests.

In agriculture, mainly two types (solids and liquids) of microorganism-based products or bioformulations are used. In solid-based bioformulations, granules, wettable powders, water-dispersible granules are mainly used. Granules are dry particles that include the active substance (microorganisms or their products), as well as a binder and a carrier. Peat, compost, agro-industrial wastes, vermiculite, perlite, rock phosphate, and polysaccharides are among commonly used solid carriers in bioformulation manufacture. MET52 ® is a type of granular bioproducts, and it is a formulation of fungi *M. anisopliae* which is used in order to control black vine weevil larvae [132]. *Trichoderma* is well-known for its beneficial role in agriculture, and almost 55.3% of *Trichoderma* formulations are commercialized as wettable powders [133]. Powdery mildew is caused by numerous pathogenic species in agriculture and *Ampelomyces quisqualis* fungi when water-dispersible granules have the ability to control this disease. Representative fungi used in solid bioformulations are *Ampelomyces quisqualis* (AQ 10 WG), *Coniothyrium minitans* (Contans WG), *Clonostachys rosea* (Prestop and Biobest), and *Trichoderma* species (Trichox WP, Biox, and Binab T) [134]. Commercial products comprising fungal isolates are presented in Table 3.5. Due to its innumerable benefits, solid-state fermentation (SSF) has received a lot of attention in recent years. SSF facilitates co-cultivation of two microorganisms, enrichment with soluble P, induction of biocontrol activity, and use of solid substrates either alone or combined, and moistened with liquid wastes [135]. Microorganisms when used as liquid bioformulations improve cell suspension viscosity, stability, and dispersion capacity achieved by water and oils using a combination of fraction [136]. The difficulty with liquid bioformulations is that the microbial population and metabolic activity rapidly decline once cell suspensions are introduced into the soil, especially if they do not contain appropriate additives. Trichojet, Enpro-Derma, and Trichorich-L are some of the *Trichoderma*-based liquid formulations (Table 3.5) [137].

Table 3.5 Fungi-based commercial products used in agriculture

Commercial fungus-based products used in agriculture	Uses	Company
MycoApply Diehard Pro-Mix	Enhance plant growth and development and phosphorus content	Mycorrhizal Applications (Grants Pass, OR); Horticultural Alliance (Sarasota, FL); Premier Tech (Rivière-du-Loup, Canada)
Nitragin Gold ZHO N-Dure Twin N	Nitrogen fixation	Novozymes BioAg (Brookfield, WL); Botanicare (Chandler, AZ) IN TX Microbials, LLC (Kentland, IN); Active Soil (Evesham, UK)
MaxQ Arkplus AR1 or AR37	Provide biotic and abiotic stress resistance	Pennington Seed Co. (Madison, GA); FFR Cooperative (Lafayette, IN); AgResearch Grasslanz (Palmerton North, NZ)
Topshield, Rootshield	Biocontrol	Bioworks, Geneva, NY
T-22G, T-22B	Biocontrol	TGT Inc., New York
Harzian 20, Harzian 10	Biocontrol	Natural Plant Protection, Nogueres, France
Supraavit	Biocontrol	Bonegaard and Reitzel, Denmark
Bio bus 1,00WP	Biocontrol	Nam Bac, Vietnam
BioSpark Trichoderma	Biocontrol	Biospark Corporation, Philippines
Trichoguard-WP, Trichoguard-L, Bio-Dart	Biocontrol	Ajay Bio-Tech (I) Limited, Pune
Tricho-Card, NIPROT, NIPROT	Biocontrol	Biocontrol Research Laboratories, Karnataka
Biolep, Bioderma, Biomonas, Biogramma	Biocontrol	Biotech International Limited, New Delhi
Biovidi, Biozim, Bionizer, Sachcer	Biocontrol	Bioved Research and Communication Centre, Uttar Pradesh
Esvin Pseudo, Biolarvex, Biogrubex, Biosappex, Eswin Tricho	Biocontrol	Esvin Advanced Technologies Limited, Tamil Nadu
Sanjeemni, WP P-Suraksha, WP Daman	Biocontrol	International Panaacea Limited, New Delhi
Prestop and Biobest	Fungicide	Adjuvant Plus Inc. (Endofine®)
CONTANS WG	Biofungicide	Prophyta company (Malchow, Germany)
JumpStart®, TagTeam®, and N-Prove®	Biofertility (nitrogen fixing and phosphate solubilizing)	Novozymes Biologicals BioAg Group
MET52	Bioinsecticide	Novozymes Biologicals BioAg Group

Sources: Morton et al., 2014 [138]; Pandya et al., 2014 [139]; Bejarano and Puopolo 2020 [134]

3.4 Conclusions

Soil health has a significant impact on environmental sustainability, including agriculture. Soil health is mainly affected by soil microorganisms which balance soil quality, soil detoxification, and soil health restoration and also maintain plant health. The chapter presents the mechanism by which the fungi play a critical role in ameliorating soil quality including physical, chemical, and biological properties. The pivotal role of *Trichoderma* in biocontrol, plant growth promotion, and enhancing soil quality has been represented. Laccase, cytochrome P450, and LiP are the key enzymes involved in degradation of environmental pollutants. Multiple functions of fungi as shown in the chapter emphasize their involvement in soil detoxification, soil building, and soil health restoration. Because of which they represented an irreplaceable segment of soil microbial diversity. Commercial fungal products to improve soil quality, plant growth, and biocontrol activity are discussed in this chapter. However, products for remediation of environmental pollutants are not common in the market. There is a huge scope for research in this area. More research is needed to determine the optimum method for preserving fungal biodiversity in soil, taking into account fungal roles and ecosystem services as disease control, contaminant detection, and bioremediation. It's critical to have the correct tools and be able to identify species as well as characterize their environmental role.

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References

1. Hao J, Ashley K (2021) Irreplaceable role of amendment-based strategies to enhance soil health and disease suppression in potato production. Microorganisms 9:1660
2. Tahat M, Alananbeh M, Othman KA, Leskovar I (2020) Soil health and sustainable agriculture. Sustainability 12(12):4859
3. Harman G, Khadka R, Doni F, Uphoff N (2021) Benefits to plant health and productivity from enhancing plant microbial symbionts. Front Plant Sci 11:610065
4. Li J, Meng B, Chai H, Yang X, Song W, Li S, Lu A, Zhang T, Sun W (2019) Arbuscular mycorrhizal fungi alleviate drought stress in C3 (*Leymus chinensis*) and C4 (*Hemarthria altissima*) grasses via altering antioxidant enzyme activities and photosynthesis. Front Plant Sci 10:499
5. Tarin MWK, Fan L, Xie D, Tayyab M, Rong J, Chen L, Muneer MA, Zheng Y (2021) Response of soil fungal diversity and community composition to varying levels of bamboo biochar in red soils. Microorganisms 9(7):1385
6. Jeffries P, Gianinazzi S, Perotto S, Turnau K, Barea JM (2003) The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biol Fertil Soils 37(1):1–16
7. Jiménez-González MA, De la Rosa JM, Jiménez-Morillo NT, Almendros G, González-Pérez JA, Knicker H (2016) Post-fire recovery of soil organic matter in a Cambisol from typical Mediterranean forest in southwestern Spain. Sci Total Environ 572:1414–1421

8. Mubekaphi C (2019) Soil organic carbon, glomalin related soil protein and related physical properties after 15 years of different management practices in a subtropical region of South Africa (doctoral thesis). University of KwaZulu-Natal, Pietermaritzburg, South Africa
9. Dellagi A, Quillere I, Hirel B (2020) Beneficial soil-borne bacteria and fungi: a promising way to improve plant nitrogen acquisition. J Exp Bot 71(15):4469–4479
10. Li JT, Lu JL, Wang HY, Fang Z, Wang XJ, Feng SW, Liang JL (2021) A comprehensive synthesis unveils the mysteries of phosphate-solubilizing microbes. Biol Rev 96(6): 2771–2793
11. Gautam AK & Avasthi S (2019) Fungal endophytes: potential biocontrol agents in agriculture. In Role of plant growth promoting microorganisms in sustainable agriculture and nanotechnology, Woodhead Publishing, pp. 241–283
12. Ali WA, Hussein RH, Radef WT (2021) The effect of soil properties on the biological diversity of fungi in soil University of Anbar. In J Physics: Conference Series 2114(1):012068
13. FAO & ITPS (2015). Status of the World's soil resources (SWSR)—Main report. Food and agriculture Organization of the United Nations and Intergovernmental Technical Panel on Soils, Rome, Italy
14. Menta C (2012) Soil fauna diversity-function, soil degradation, biological indices, soil restoration. Biodiversity conservation and utilization in a diverse world. In Biodiversity conservation and utilization in a diverse world (eds. Gbolagade Lameed), London. pp. 59–94
15. Phogat VK, Tomar VS & Dahiya RI (2015) Soil physical properties. Soil science: An introduction pp.135–171
16. Rosas-Medina M, Maciá-Vicente JG, Piepenbring M (2020) Diversity of fungi in soils with different degrees of degradation in Germany and Panama. Mycobiology 48(1):20–28
17. Rousk J, Brookes PC, Baath E (2009) Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. Appl Environ Microbiol 75(6):1589–1596
18. Jayaswal K, Sahu V & Gurjar BR (2018). Water pollution, human health and remediation. In Water remediation. Springer, Singapore, pp. 11–27
19. Bisht N & Chauhan PS (2020) Excessive and disproportionate use of chemicals cause soil contamination and nutritional stress. In Soil Contamination-Threats and Sustainable Solutions. IntechOpen
20. Norton JM, Pellegrino E, Gamper HA, Ciccolini V, Ercoli L (2020) Forage rotations conserve diversity of arbuscular mycorrhizal fungi and soil fertility. Front Microbiol 10:2969
21. Fall AF, Nakabonge G, Ssekandi J, Founoune-Mboup H, Apori SO, Ndiaye A, Badji A, Ngom K (2022) Roles of arbuscular mycorrhizal fungi on soil fertility: contribution in the improvement of physical, chemical, and biological properties of the soil. Front Fungal Biol 3:723892
22. Frac M, Hannula SE, Bełka M, Jedryczka M (2018) Fungal biodiversity and their role in soil health. Front Microbiol 9:707
23. Lehmann A, Zheng W, Ryo M, Soutschek K, Roy J, Rongstock R, Maaß S, Rillig MC (2020) Fungal traits important for soil aggregation. Front Microbiol 10:2904
24. Wilson GW, Rice CW, Rillig MC, Springer A, Hartnett DC (2009) Soil aggregation and carbon sequestration are tightly correlated with the abundance of arbuscular mycorrhizal fungi: results from long-term field experiments. Ecol Lett 12(5):452–461
25. Zhu C, Ling N, Guo J, Wang M, Guo S, Shen Q (2016) Impacts of fertilization regimes on arbuscular mycorrhizal fungal (AMF) community composition were correlated with organic matter composition in maize rhizosphere soil. Front Microbiol 7:1840
26. Zhang L, Xu M, Liu Y, Zhang F, Hodge A, Feng G (2016) Carbon and phosphorus exchange may enable cooperation between an arbuscular mycorrhizal fungus and a phosphate-solubilizing bacterium. New Phytol 210(3):1022–1032
27. Jansa J, Forczek ST, Rozmoš M, Püschel D, Bukovská P, Hršelová H (2019) Arbuscular mycorrhiza and soil organic nitrogen: network of players and interactions. Chem Biol Technol Agric 6(1):1–10

28. Xu J, Liu S, Song S, Guo H, Tang J, Yong JW, Ma Y, Chen X (2018) Arbuscular mycorrhizal fungi influence decomposition and the associated soil microbial community under different soil phosphorus availability. Soil Biol Biochem 120:181–190
29. Zhang L, Shi N, Fan J, Wang F, George TS, Feng G (2018) Arbuscular mycorrhizal fungi stimulate organic phosphate mobilization associated with changing bacterial community structure under field conditions. Environ Microbiol 7:2639–2651
30. Zhu J, Li M, Whelan M (2018) Phosphorus activators contribute to legacy phosphorus availability in agricultural soils: a review. Sci Total Environ 612:522–537
31. Rodrigues M, Withers PJA, Soltangheisi A, Vargas V, Holzschuh M, Pavinato PS (2021) Tillage systems and cover crops affecting soil phosphorus bioavailability in Brazilian Cerrado Oxisols. Soil Till Res 205:104770
32. Choudhary M, Sharma PC, Jat HS, McDonald A, Jat ML, Choudhary S, Garg N (2018) Soil biological properties and fungal diversity under conservation agriculture in Indo-Gangetic Plains of India. J Plant Nutr Soil Sci 18(4):1142–1156
33. Kour D, Rana KL, Kaur T, Yadav N, Halder SK, Yadav AN et al (2020) Potassium solubilizing and mobilizing microbes: biodiversity, mechanisms of solubilization, and biotechnological implication for alleviations of abiotic stress. In: Rastegari AA, Yadav AN, Yadav N (eds) New and Future Developments in Microbial Biotechnology and Bioengineering. Elsevier, pp 177–202
34. Albertsen A, Ravnskov S, Green H, Jensen DF, Larsen J (2006) Interactions between the external mycelium of the mycorrhizal fungus *Glomus intraradices* and other soil microorganisms as affected by organic matter. Soil Biol Biochem 38(5):1008–1014
35. Welc M, Ravnskov S, Kieliszewska-Rokicka B, Larsen J (2010) Suppression of other soil microorganisms by mycelium of arbuscular mycorrhizal fungi in root-free soil. Soil Biol Biochem 42:1534–1540. https://doi.org/10.1016/j.soilbio.2010.05.024
36. Gryndler M (2000) Interactions of arbuscular mycorrhizal fungi with other soil organisms. In: Kapulnik Y, Douds DD (eds) Arbuscular mycorrhizas. Physiology and function. Springer, Dordrecht, pp 239–262
37. Verbruggen E, Van Der HEIJDENMG, Weedon JT, Kowalchuk GA, Röling WF (2012) Community assembly, species richness and nestedness of arbuscular mycorrhizal fungi in agricultural soils. Mol Ecol 21(10):2341–2353
38. Hamel C, Plenchette C (2017) Implications of past, current, and future agricultural practices for mycorrhiza-mediated nutrient flux. In: Johnson NC, Gehring C, Jansa J (eds) Mycorrhizal mediation of soil. Elsevier, Amsterdam, pp 175–186
39. Bhandari G (2014) An overview of agrochemicals and their effects on environment in Nepal. Appl Ecol Environ Sci 2(2):66–73
40. Kumari A & Sundari SK (2022) *In vitro* and *in vivo* evidence for the mitigation of monocrotophos toxicity using native isolate of *Trichoderma harzianum* (In Press, Biologia journal, Springer)
41. Fasusi OA, Cruz C, Babalola OO (2021) Agricultural sustainability: microbial biofertilizers in rhizosphere management. Agriculture 11(2):163
42. Abdel-Kareem MM, Zohri AN, Nasr SA (2021) Novel marine yeast strains as plant growth-promoting agents improve defense in wheat (*Triticum aestivum*) against *fusarium oxysporum*. J Plant Dis Prot 128(4):973–988
43. Murali M, Naziya B, Ansari MA, Alomary MN, AlYahya S, Almatroudi A, Thriveni MC, Gowtham HG, Singh SB, Aiyaz M, Kalegowda N (2021) Bioprospecting of rhizosphere-resident fungi: their role and importance in sustainable agriculture. J Fungi 7(4):314
44. Omotayo OP, Babalola OO (2021) Resident rhizosphere microbiome's ecological dynamics and conservation: towards achieving the envisioned sustainable development goals, a review. Int Soil Water Conserv Res 9(1):127–142
45. Rashid MI, Mujawar LH, Shahzad T, Almeelbi T, Ismail IM, Oves M (2016) Bacteria and fungi can contribute to nutrients bioavailability and aggregate formation in degraded soils. Microbiol Res 183:26–41

46. Liermann LJ, Hausrath EM, Anbar AD, Brantley SL (2007) Assimilatory and dissimilatory processes of microorganisms affecting metals in the environment. J Anal At Spectrom 22(8): 867–877
47. Fenice M (2021) The nitrogen cycle: an overview. Nitrogen Cycle: Ecology, Biotechnological Applications and Environmental Impacts 22(1):1–21
48. Akhtar M, Sarwar N, Ashraf A, Ejaz A, Ali S, Rizwan M (2021) Beneficial role of *Azolla sp.* in paddy soils and their use as bioremediators in polluted aqueous environments: implications and future perspectives. Arch Agron Soil Sci 67(9):1242–1255
49. Gough EC, Owen KJ, Zwart RS, Thompson JP (2021) Arbuscular mycorrhizal fungi acted synergistically with *Bradyrhizobium sp.* to improve nodulation, nitrogen fixation, plant growth and seed yield of mung bean (*Vigna radiata*) but increased the population density of the root-lesion nematode *Pratylenchus thornei*. Plant Soil 465(1):431–452
50. Ingraffia R, Amato G, Frenda AS, Giambalvo D (2019) Impacts of arbuscular mycorrhizal fungi on nutrient uptake, N2 fixation, N transfer, and growth in a wheat/*faba bea*n intercropping system. PLoS One 14(3):e0213672
51. Wang HW, Ma CY, Xu FJ, Lu F, Zhang W, Dai CC (2021) Root endophyte-enhanced peanut-rhizobia interaction is associated with regulation of root exudates. Microbiol Res 250:126765
52. Rawat P, Das S, Shankhdhar D, Shankhdhar SC (2021) Phosphate-solubilizing microorganisms: mechanism and their role in phosphate solubilization and uptake. J Soil Sci Plant Nutr 21(1):49–68
53. Emami-Karvani Z & Chitsaz-Esfahani Z (2021) Phosphorus solubilization: mechanisms, recent advancement and future challenge. In: Soil microbiomes for sustainable agriculture. Springer, Cham, pp. 85–131
54. Wang J, Zhao YG, Maqbool F (2021) Capability of *penicillium oxalicum* y2 to release phosphate from different insoluble phosphorus sources and soil. Folia Microbiol 66(1):69–77
55. Pandey D, Kehri HK, Zoomi I, Singh U, Chaudhri KL & Akhtar O (2020). Potassium solubilizing microbes: diversity, ecological significances and biotechnological applications. In Plant Microbiomes Sust Agr, Springer, Cham, pp. 263–286
56. Sattar A, Naveed M, Ali M, Zahir ZA, Nadeem SM, Yaseen M, Meena HN (2019) Perspectives of potassium solubilizing microbes in sustainable food production system: a review. Appl Soil Ecol 133:146–159
57. Pathak A, Kothari R, Vinoba M, Habibi, Tyagi VV (2021) Fungal bioleaching of metals from refinery spent catalysts: a critical review of current research, challenges, and future directions. J Environ Manag 280:111789
58. Muthuraja R, Muthukumar T (2021) Isolation and characterization of potassium solubilizing *aspergillus* species isolated from saxum habitats and their effect on maize growth in different soil types. Geomicrobiol J 38:672–685
59. Zenda T, Liu S, Dong A, Duan H (2021) Revisiting Sulphur—the once neglected nutrient: its roles in plant growth, metabolism, stress tolerance and crop production. Agriculture 11(7):626
60. Kawa D (2021) Outsourcing transport: sulfate delivery by an endophytic fungus to maize, pp. 1095-1096
61. Narayan OP, Kumar P, Yadav B, Dua M, Johri AK (2022) Sulfur nutrition and its role in plant growth and development. Plant Signal Behav:2030082
62. Linder T (2018) Assimilation of alternative sulfur sources in fungi. World J Microbiol Biotechnol 34(4):1–7
63. Miethke M, Marahiel MA (2007) Siderophore-based iron acquisition and pathogen control. Microbiol Mol Biol Rev 71(3):413–451
64. Pecoraro L, Wang X, Shah D, Song X, Kumar V, Shakoor A, Rani R (2021) Biosynthesis pathways, transport mechanisms and biotechnological applications of fungal siderophores. J Fungi 8(1):21
65. Manoharan S, Ramalakshmi OI & Ramasamy S (2021). Fungal siderophores: prospects and applications. In Fungal Siderophores Springer, Cham, pp. 141–156

66. Haas H (2014) Fungal siderophore metabolism with a focus on *Aspergillus fumigatus*. Nat Prod Rep 31(10):1266–1276
67. Rütting T, Boeckx P, Müller C, Klemedtsson L (2011) Assessment of the importance of dissimilatory nitrate reduction to ammonium for the terrestrial nitrogen cycle. Biogeosciences 8(7):1779–1791
68. Coyne MS, Lal R, Stewart BA (2018) Denitrification in soil. Soil Nitrogen Uses and Environmental Impacts 1:95–139
69. Higgins SA, Schadt CW, Matheny PB, Löffler FE (2018) Phylogenomics reveal the dynamic evolution of fungal nitric oxide reductases and their relationship to secondary metabolism. Genome Boil Evol 10(9):2474–2489
70. Ruiz-Herrera J, Starkey RL (1969) Dissimilation of methionine by fungi. J Bacteriol 99(2): 544–551
71. Raut RR, Harale P, Kurhe A (2020) Studies on soil quality parameters in relations to cropping patterns, micronutrients and pH from Goagalgaon area in Ahmednagar District of Maharashtra. India Int J Multidiscip Curr Res 6:210–219
72. Ashraf MA, Maah MJ, Yusoff I (2014) Soil contamination, risk assessment and remediation. Environ Risk Assess Soil Contam 25:3–56
73. Ahmad I, Imran M, Hussain MB & Hussain S (2017) Remediation of organic and inorganic pollutants from soil: the role of plant bacteria partnership. Chemical Pollution Control with Microorganisms 197–243
74. Burgess L (2013) The effects of organic pollutants in soil on human health. In EGU General Assembly Conference Abstracts, pp. 979
75. Sundari SK, Prakash A, Yadav P, Kumari A (2019) Plant growth-promoting microbes as front-runners for on-site remediation of organophosphate pesticide residues in agriculture soils. In: Arora N, Kumar N (eds) Phyto and Rhizo remediation. Microorganisms for sustainability, vol 9. Springer, Singapore, pp 249–285
76. Singh G, Dwivedi SK, Mishra J (2020) Role of fungal enzymes in the removal of azo dyes. In Microbial enzymes: roles and applications in industries. Springer, Singapore, pp 231–257
77. Deshmukh R, Khardenavis AA, Purohit HJ (2016) Diverse metabolic capacities of fungi for bioremediation. Indian J Microbiol 56:247–264
78. Shi Y, Yang Z, Xing L, Zhang X, Li X, Zhang D (2021) Recent advances in the biodegradation of azo dyes. World J Microbiol Biotechnol 37(8):1–18
79. Ortiz-Hernández ML, Sánchez-Salinas E, Dantán-González E, Castrejón-Godínez ML (2013) Pesticide biodegradation: mechanisms, genetics and strategies to enhance the process. Biodegrad Life Sci:251–287
80. Huang Y, Xiao L, Li F, Xiao M, Lin D, Long X, Wu Z (2018) Microbial degradation of pesticide residues and an emphasis on the degradation of cypermethrin and 3-phenoxy benzoic acid: a review. Molecules 23(9):2313
81. Hu K (2021) Developing and scaling up a trickle bed reactor for degrading pesticides from agricultural wastewater by fungi (Doctoral dissertation, Universitat Autònoma de Barcelona)
82. Henn C, Monteiro DA, Boscolo M, Da Silva R, Gomes E (2020) Biodegradation of atrazine and ligninolytic enzyme production by basidiomycete strains. BMC Microbiol 20(1):1–12
83. Mohanan N, Montazer Z, Sharma PK, Levin DB (2020) Microbial and enzymatic degradation of synthetic plastics. Front Microbiol 11:580709
84. Saravanan A, Kumar PS, Vo DV, Jeevanantham S, Karishma S, Yaashikaa PR (2021) A review on catalytic-enzyme degradation of toxic environmental pollutants: microbial enzymes. J Hazard Mater 419:126451
85. Yoshida S, Hiraga K, Takehana T, Taniguchi I, Yamaji H, Maeda Y, Toyohara K, Miyamoto K, Kimura Y, Oda K (2016) A bacterium that degrades and assimilates poly (ethylene terephthalate). Science 351(6278):1196–1199
86. Pan L, Fang G, Wang Y, Wang L, Su B, Li D, Xiang B (2018) Potentially toxic element pollution levels and risk assessment of soils and sediments in the upstream river, Miyun reservoir, China. Intel J Environ Res Pub Health 15(11):2364

87. Gogoi NM, Baroowa B, Gogoi N (2021) Ecological tools for remediation of soil pollutants. In Bioremediation science from theory to practice. CRC Press, pp 57–78
88. Verma A (2021) Bioremediation techniques for soil pollution: an introduction in P. In: Mendes KF, de Sousa RN, Mielke KC (eds) Biodegradation Technology of Organic and Inorganic Pollutants. IntechOpen, London. https://doi.org/10.5772/intechopen.99028
89. Kumar V, Dwivedi SK (2021) Mycoremediation of heavy metals: processes, mechanisms, and affecting factors. Environ Sci Pollut Res 28(9):10375–10412
90. Khan I, Aftab M, Shakir S, Ali M, Qayyum S, Rehman MU, Haleem KS, Touseef I (2019) Mycoremediation of heavy metal (Cd and Cr)–polluted soil through indigenous metallotolerant fungal isolates. Environ Monit Assess 191(9):1–1
91. Derco J, Vrana B (2018) In: Derco J, Vrana B (eds) Biosorption. IntechOpen
92. Kumari R, Singh A & Yadav AN (2021) Fungal enzymes: degradation and detoxification of organic and inorganic pollutants. In Recent trends in mycological research. Springer, Cham, pp. 99–125
93. Singh NS, Sharma R, Singh DK (2019) Identification of enzyme (s) capable of degrading endosulfan and endosulfan sulfate using in silico techniques. Enzym Microb Technol 124:32–40
94. Da Silva NA, Birolli WG, Seleghim MH, Porto AL (2013) Biodegradation of the organophosphate pesticide Profenofos by marine fungi. Appl Bioremed Active Passive Approc 2:149–180
95. Williams MM II, Boydston RA, Peachey RE, Robinson D (2011) Performance consistency of reduced atrazine use in sweet corn. Field Crops Res 121(1):96–104
96. Sakaki T, Yamamoto K, Ikushiro S (2013) Possibility of application of cytochrome P450 to bioremediation of dioxins. Biotechnol Appl Biochem 60(1):65–70
97. Čvančarová M, Křesinová Z, Filipová A, Covino S, Cajthaml T (2012) Biodegradation of PCBs by ligninolytic fungi and characterization of the degradation products. Chemosphere 11: 1317–1323
98. Rosales E, Pazos M, Ángeles Sanromán M (2013) Feasibility of solid-state fermentation using spent fungi-substrate in the biodegradation of PAHs. Clean Soil Air Water 41:610–615
99. Pazarlioglu NK, Akkaya A, Akdogan HA, Gungor B (2010) Biodegradation of direct blue 15 by free and immobilized *Trametes versicolor*. Water Environ Res 7:579–585
100. Couto SR, Rosales E, Sanromán MA (2006) Decolourization of synthetic dyes by *Trametes hirsuta* in expanded-bed reactors. Chemosphere 62(9):1558–1563
101. Pandi A, Kuppuswami GM, Ramudu KN, Palanivel SA (2019) Sustainable approach for degradation of leather dyes by a new fungal laccase. J Clean Prod 211:590–597
102. Bankole PO, Adekunle AA, Obidi OF, Chandanshive VV, Govindwar SP (2018) Biodegradation and detoxification of scarlet RR dye by a newly isolated filamentous fungus, *Peyronellaea prosopidis*. Sustain Environ Res 28(5):214–222
103. He XL, Song C, Li YY, Wang N, Xu L, Han X, Wei DS (2018) Efficient degradation of Azo dyes by a newly isolated fungus *Trichoderma tomentosum* under non-sterile conditions. Ecotoxicol Environ Saf 150:232–239
104. Chakraborty S, Mukherjee A, Das TK (2013) Biochemical characterization of a lead-tolerant strain of *Aspergillus foetidus*: an implication of bioremediation of lead from liquid media. Int Biodeterior Biodegradation 84:134–142
105. Muneer B, JIqbal M, Shakoori FR, Shakoori AR (2013). Tolerance and biosorption of mercury by microbial consortia: potential use in bioremediation of wastewater. Pak J Zool 45: 247–254
106. Parvathi K, Nagendran R (2007) Biosorption of chromium from effluent generated in chrome-electroplating unit using Saccharomyces cerevisiae. Sep Sci Technol 42(3):625–638
107. Taştan BE, Ertuğrul S, Dönmez G (2010) Effective bioremoval of reactive dye and heavy metals by *Aspergillus versicolor*. Bioresour Technol 101(3):870–876
108. Mitra A, Roy D, Roy P, Bor AM, Sarkar Mitra AK (2014) Sustainability of Aspergillus spp. in metal enriched substrate aiming towards increasing bioremediation potential. World J Pharm Pharm Sci 3:864–878

109. Russell JR, Huang J, Anand P, Kucera K, Sandoval AG, Dantzler KW, Hickman D, Jee J, Kimovec FM, Koppstein D, Marks DH (2011) Biodegradation of polyester polyurethane by endophytic fungi. Appl Environ Microbiol 77(17):6076–6084
110. Brunner I, Fischer M, Rüthi J, Stierli B, Frey B (2018) Ability of fungi isolated from plastic debris floating in the shoreline of a lake to degrade plastics. PLoS One 13(8):e0202047
111. Ojha N, Pradhan N, Singh S, Barla A, Shrivastava A, Khatua P, Rai V, Bose S (2017) Evaluation of HDPE and LDPE degradation by fungus, implemented by statistical optimization. Sci Rep 7(1):1–3
112. Jung HW, Yang MK, Su RC (2018) Purification, characterization, and gene cloning of an *aspergillus fumigatus* polyhydroxybutyrate depolymerase used for degradation of polyhydroxybutyrate, polyethylene succinate, and polybutylene succinate. Polym Degrad Stab 154:186–194
113. Zuo LZ, Li HX, Lin L, Sun YX, Diao ZH, Liu S, Zhang ZY, Xu XR (2019) Sorption and desorption of phenanthrene on biodegradable poly(butylene adipate co-terephtalate) microplastics. Chemosphere 215:25–32
114. Kaushal J, Khatri M, Arya SK (2021) Recent insight into enzymatic degradation of plastics prevalent in the environment: a mini-review. Clean Eng Technol 2:100083
115. Hu X, Su T, Li P, Wang Z (2018) Blending modification of PBS/PLA and its enzymatic degradation. Polym Bull 75(2):533–546
116. Hegyesi N, Zhang Y, Kohari A, Polyák P, Sui X, Pukanszky B (2019) Enzymatic degradation of PLA/cellulose nanocrystal composites. Ind Crop Prod 141:111799
117. Guhra T, Stolze K, Totsche KU (2022) Pathways of biogenically excreted organic matter into soil aggregates. Soil Biol Biochem 164:108483
118. Hannula SE, Morriën E (2022) Will fungi solve the carbon dilemma? Geoderma 413:115767
119. Witzgall K, Vidal A, Schubert DI, Hoschen C, Schweizer SA, Buegger F, Pouteau V, Chenu C, Mueller CW (2021) Particulate organic matter as a functional soil component for persistent soil organic carbon. Nat Commun 12(1):1–10
120. Frąc M (2015) Occurrence, detection, and molecular and metabolic characterization of heat-resistant fungi in soils and plants and their risk to human health. Adv Agron 132:161–204
121. Chanclud E, Morel JB (2016) Plant hormones: a fungal point of view. Mol Plant Pathol 17(8): 1289–1297
122. Ellouze W, Esmaeili Taheri A, Bainard LD, Yang C, Bazghaleh N, Navarro-Borrell A, Hanson K, Hamel C. (2014) Soil fungal resources in annual cropping systems and their potential for management. BioMed. Res. Intel. pp. 2014
123. Senthil-Nathan S (2015) A review of biopesticides and their mode of action against insect pests. Environment Sustain:49–63
124. Devi R, Kaur T, Kour D, Rana KL, Yadav A, Yadav AN (2020) Beneficial fungal communities from different habitats and their roles in plant growth promotion and soil health. Microbial Biosyst 5(1):21–47
125. Cai F, Yu G, Wang P, Wei Z, Fu L, Shen Q, Chen W (2013) Harzianolide, a novel plant growth regulator and systemic resistance elicitor from *Trichoderma harzianum*. Plant Physiol Biochem 73:106–113
126. Rani A, Saini KC, Bast F, Mehariya S, Bhatia SK, Lavecchia R, Zuorro A (2021) Microorganisms: a potential source of bioactive molecules for antioxidant applications. Molecules 26(4):1142
127. Pusztahelyi T, Holb IJ, Pócsi I (2015) Secondary metabolites in fungus-plant interactions. Front Plant Sci 6:573
128. Tijjani A, Bashir K, Mohammed I, Muhammad A, Gambo A, Musa H (2016) Biopesticides for pests control: a review. J Biopest Agric 3(1):6–13
129. Saxena J, Chandra S, Nain L (2013) Synergistic effect of phosphate solubilizing rhizobacteria and arbuscular mycorrhiza on growth and yield of wheat plants. J Soil Sci Plant Nutr 13(2): 511–525

130. Woo S, Fogliano V, Scala F, Lorito M (2002) Synergism between fungal enzymes and bacterial antibiotics may enhance biocontrol. Antonie Leeuwenhoek 81(1):353–356
131. Barea JM, Azcon R, Azcon-Aguilar C (2005) Interactions between mycorrhizal fungi and bacteria to improve plant nutrient cycling and soil structure. In Microorganisms in soils: roles in genesis and functions. Springer, Berlin, Heidelberg, pp. 195–212
132. Ansari MA, Butt TM (2012) Susceptibility of different developmental stages of large pine weevil *Hylobius abietis* (Coleoptera: Curculionidae) to entomopathogenic fungi and effect of fungal infection to adult weevils by formulation and application methods. J Invertebr Pathol 111(1):33–40
133. Woo SL, Ruocco M, Vinale F, Nigro M, Marra R, Lombardi N, Pascale A, Lanzuise S, Manganiello G, Lorito M (2014) *Trichoderma*-based products and their widespread use in agriculture. Open Mycol J 8(1):71–126
134. Bejarano A, Puopolo G (2020) Bioformulation of microbial biocontrol agents for a sustainable agriculture. In: CalA D, Melgarejo P, Magan N (eds) How research can stimulate the development of commercial biological control against plant diseases. Springer, Cham, pp 275–293
135. Vassilev N, Vassileva M, Martos V, Garcia del Moral LF, Kowalska J, Tylkowski B, Malusá E (2020) Formulation of microbial inoculants by encapsulation in natural polysaccharides: focus on beneficial properties of carrier additives and derivatives. Front Plant Sci 11:270
136. Bashan Y, de-Bashan LE, Prabhu SR (2016) Superior polymeric formulations and emerging innovative products of bacterial inoculants for sustainable agriculture and the environment. In: Singh HB, Sarma BK, Keswani C (eds) Agriculturally important microorganisms - commercialization and regulatory requirements in Asia. Springer, Berlin, pp 15–46
137. Mishra J & Arora NK (2016) Bioformulations for plant growth promotion and combating phytopathogens: a sustainable approach. In Bioformulations: for sustainable agriculture, Springer, New Delhi, pp. 3–33
138. Morton JB, Benedito VA, Panaccione DG, Jenks MA (2014) Potential for industrial application of microbes in symbioses that influence plant productivity and sustainability in agricultural, natural, or restored ecosystems. Ind Biotechnol 10(5):347–353
139. Pandya U & Saraf M (2014) In vitro evaluation of PGPR strains for their biocontrol potential against fungal pathogens. In Plant Microbes Symbiosis: Applied Facets. pp. 179–192

Emerging Mucormycosis: Problems and Treatments

4

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Abstract

Fungi of the order *Mucorales* and class *Zygomycetes* cause mucormycosis that is opportunistic and rapidly emerging. In recent years, an increase in cases has been observed in the healthcare setting, mainly in immunocompromised patients especially those with diabetes mellitus. The current challenge is a spike in mucormycosis cases in COVID-19 patients even post recovery. The elements fundamental for successful treatment are early diagnosis, management of predisposing factors, debridement of infected tissues surgically, and adequate antifungal therapy. The gold standard for diagnosis is histopathology in correlation with clinical features and culture findings. Multiple molecular methods for detecting newer targets are under evaluation. The drug of choice for treatment is amphotericin B, whereas for salvage therapy, posaconazole or isavuconazole may be used.

Keywords

Mucormycosis · Immunocompromised patients · COVID-19 · Rapid diagnosis · Amphotericin B · Salvage therapy

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4.1 Introduction

Agents causing mucormycosis are distributed worldwide and are among the pathogens resulting in invasive fungal infections with an increase in incidence [1]. The third most common invasive fungal infection is mucormycosis, following aspergillosis and candidiasis, and accounts for 8.3–13% of all fungal infections diagnosed at autopsy in hematology patients [2]. The causative agents are fungi of the family *Mucoraceae* belonging to order *Mucorales*. The common genera, namely, *Rhizopus*, *Mucor*, and *Lichtheimia* (earlier *Absidia*), account for 70–80% of the cases, while <1–5% of reported cases are caused by *Cunninghamella*, *Apophysomyces*, *Saksenaea*, *Rhizomucor*, *Cokeromyces*, and *Syncephalastrum*. A characteristic feature of the *Mucorales* is that they can cause very serious infections with high morbidity and mortality even in relatively immunocompetent individuals, e.g., apart from patients with diabetes mellitus or neonates, those with wounds caused by surgery, trauma, and burns can also be affected. In spite of adequate surgical intervention and intensive antifungal treatment, the mortality of mucormycosis is high, ranging from about 50 to 100% depending on the form of disease. In contrast, 20–50% and 35–45% are the mortality rates for candidiasis and aspergillosis, respectively. Early diagnosis, treatment of underlying predisposing risk factors, surgical management, and timely administration of amphotericin B are required for successful management of mucormycosis. However, in spite of such aggressive measures, mucormycosis may not always be cured [1].

4.2 Microbiology

Fungi of the order *Mucorales* are as mentioned below in Table 4.1

The agents of mucormycosis have a varied geographical distribution. In European studies, the most commonly found were *Rhizopus* spp., followed by *Lichtheimia* spp. and *Mucor* spp. [5, 6]. In India, the second most commonly isolated were *Apophysomyces* spp. [7, 8]. *Rhizopus homothallicus*, *Thamnostylum lucknowense*, *Mucor irregularis*, and *Saksenaea erythrospora* are few of the newer emerging species [9].

Mucorales show rapid and good growth on both nonselective and selective media. The mycelial elements are fibrous and hence called "cotton candy-like." The growth is so vigorous that it covers the entire plate in only a few days which gives the group the name of "lid lifters." The agents responsible for mucormycosis are identified by both macroscopic and microscopic morphology, assimilation of carbohydrates, and maximum temperature conducive for their growth.

A presumptive identification can be made based on macroscopic criteria that need to be confirmed by microscopic analysis post staining. Macroscopic features such as hyaline appearance, rapid and heavy growth, reverse side of the plate showing light coloration (tan to yellow most commonly), and sporulating or obverse surface showing variable degrees of coloration (pure white to tan, brown, gray, or even black) are characteristic of *Mucorales*, as in Fig. 4.1. Microscopy aids in speciation

Table 4.1 *Zygomycetes* causing human infections

Class, order	*Zygomycetes*, *Mucorales*	
Family	*Mucoraceae*	*Lichtheimiaceae*
Genus	*Apophysomyces*	*Lichtheimia* (formerly *Absidia*)
Species	*A. elegans*	*L. corymbifera*
	Mucor	*L. ramosa*
	M. circinelloides	*L. ornata*
	M. hiemalis	Cunninghamellaceae
	M. racemosus	*Cunninghamella*
	M. ramosissimus	*C. bertholletiae*
	M. rouxianus	*Mortierellaceae*
	Rhizomucor	*Mortierella* (animal pathogen)
	R. pusillus	*Saksenaceae*
	R. Miehei (animal pathogen)	*Saksenaea*
	Rhizopus	*S. vasiformis*
	R. arrhizus/oryzae	*Syncephalastraceae*
	R. azygosporus	*Syncephalastrum*
	R. microsporus	*S. racemosum*
	var. *microsporus*	*Thamnidaceae*
	var. *oligosporus*	*Cokeromyces*
	var. *rhizopodiformis*	*C. recurvatus*
	R. schipperae	
	R. stolonifer	
	R. delemar	

Adapted from Ribes et al. [3, 4]

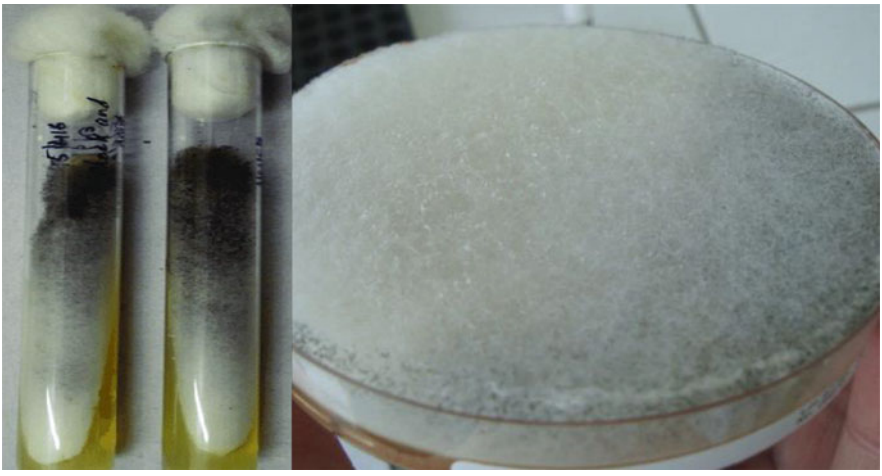

Fig. 4.1 Macroscopic appearance of *Mucorales* (Sabouraud's dextrose agar)

which is based on demonstrating important fungal elements. Various elements such as rhizoids, stolons, and columella, which are usually visualized on lactophenol cotton blue (LPCB)-stained slides, as in Fig. 4.2, aid in differentiation of the species.

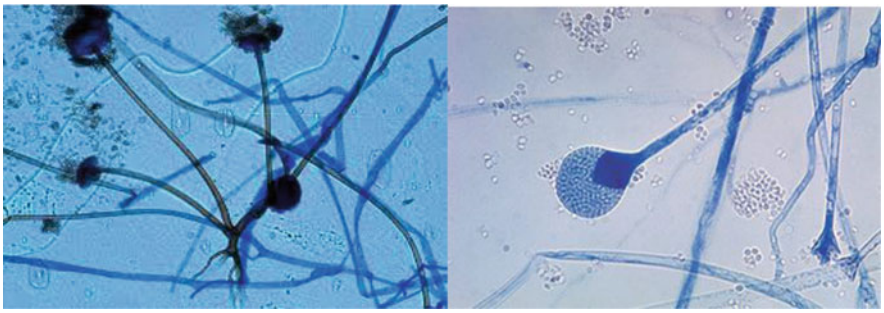

Fig. 4.2 Microscopic appearance of *Mucorales*—lactophenol cotton blue mount

4.3 Epidemiology

In recent years, mucormycosis has become an issue of utmost importance owing to a rise in cases, mortality rates that are very high, and lack of effective or ineffective antifungal treatments. Considered a rare infection in the past, being limited only to patients with severe immunocompromised conditions, it has now been shown that numerous cases have also involved immunocompetent individuals, owing to improved diagnostic techniques [10].

4.3.1 Risk Factors

Mucorales are found in soil and organic matter (decaying) and hence are ubiquitous saprophytes [2]. Mucormycosis affects patients of all ages, from premature neonates to even elderly people, who have various types of underlying conditions [1]. Malignancy (63%), diabetes (17%), and solid organ transplantation (10%) were found to be the predominant underlying conditions for mucormycosis according to a global fungal infection registry data, which studied patients from Central Europe and Asia [11]. A detailed review of the reported cases showed that diabetes (36%), malignancy (17%), solid organ transplantation (7%), deferoxamine therapy (6%), injection drug use (5%), and bone marrow transplantation (5%) were the commonly associated underlying conditions in decreasing order.

Hematological Malignancies (HM) and Hematopoietic Stem Cell Transplantation The highest risk for mucormycosis is seen in patients with acute myeloid leukemia (AML), 1–8% being the range of incidence. The frequency of mucormycosis is lower in patients with autologous or allogeneic stem cell transplantation, as compared to patients with AML, and ranges from 0.9% to 2.0%, with patients with graft-versus-host disease demonstrating the highest incidence [12].

Solid Organ Malignancies and Transplantation *Mucorales* account for a small proportion of invasive fungal infections in solid organ transplant (SOT) recipients and show a high mortality rate. The incidence may range from 0.4% to 16.0% and depends on the type of transplantation [13–16]. The incidence in renal transplant ranges from 0.2% to 1.2%, while that in liver transplants varies from 0% to 1.6%. Heart transplant patients show an incidence of 0% to 0.6%, and those with lung transplants vary from 0% to 1.5% [2]. Eighty percent of the cases in solid organ transplant recipients was seen to occur within 6 months posttransplantation, as stated by Singh et al. [17]. Pulmonary infection was more common in patients with malignancy, whereas, sino-orbital and rhinocerebral diseases were commonly seen in patients with diabetes mellitus [1]. The host's immune function along with other pathological and anatomical factors could probably be the reason for this differential spectrum [18].

Diabetes Mellitus and Ketoacidosis 36–88% of mucormycosis cases have been seen in patients with diabetes mellitus as a predisposing factor. The patient becomes more susceptible due to uncontrolled hyperglycemia especially if this coexists with ketoacidosis. In some patients with undiagnosed diabetes mellitus, mucormycosis may be the first manifestation. Patients with metabolically controlled diabetes rarely present with mucormycosis [12].

Corticosteroid Use and Rheumatic Diseases The patient's susceptibility to mucormycosis is enhanced by chronic corticosteroid-based therapy mainly due to the defects in macrophages and neutrophils and/or at times steroid-induced diabetes. Hypocomplementemia, nephrotic syndrome, uremia, leukopenia, etc. are other predisposing factors for opportunistic mucormycosis. In patients with Wegener's granulomatosis, a relapse of the underlying disease may be mimicked by mucormycosis and hence may go undiagnosed [19].

Iron Overload and Chelation Therapy with Deferoxamine Deferoxamine, also known as DFO, is an iron-chelating agent used in dialysis recipients to treat iron and/or aluminum overload. This is found to be a risk factor for angioinvasive mucormycosis [20, 21]. Apart from DFO, transfusional or dyserythropoietic iron overload by itself is a risk factor for mucormycosis [2, 12] resulting in high mortality rates of up to 80% [22].

Prolonged Use of Voriconazole The extensive use of voriconazole as a broad-spectrum antifungal agent in patients with malignancies and stem cell transplant recipients has resulted in an increase in global mucormycosis incidence in such patients by putting them at a high risk [12]. Patients receiving itraconazole prophylaxis may also be at an increased risk of mucormycosis. Patients receiving posaconazole or echinocandins prophylactically have also been shown to have breakthrough mucormycosis [3, 23, 24].

HIV or AIDS Increased risk of developing mucormycosis is not noted in cases of HIV infection, probably because T lymphocytes are involved in immunity against HIV, whereas neutrophils play a major role in defense against *Mucorales*. If at all mucormycosis occurs in an HIV patient, it is usually due to intravenous drug use [2].

No Underlying Disease or Immunocompetent Patients Patients without risk factors or immune deficiency rarely have mucormycosis. Such patients may have trauma or burns resulting in primary cutaneous mucormycosis. Mucormycosis may also be caused by iatrogenic factors such as use of catheters and other devices that may disrupt the mucocutaneous barriers. Occurrence has also been reported post exposure to fungal spores in contaminated bandages [12, 25].

Other Factors Traumatic inoculation along with water and soil contamination may also result in mucormycosis [25]. In patients with malaria, severe hemolysis, metabolic acidosis, and malaria-induced immune suppression may predispose such patients to *Mucorales* infection. Other conditions that have been associated with mucormycosis are cirrhosis, congenital heart disease, malnutrition, carcinoma, anemia, hepatitis, glomerulonephritis, uremia, amoebiasis, typhoid fever, and gastroenteritis [2].

Nosocomial Mucormycosis Construction work, air filter contamination, or contaminated procedures and devices associated with healthcare, like contaminated wound dressings, nitrate patches (transdermal), intravenous catheters, tongue depressors, allopurinol pills, etc., have been found to be associated with nosocomial mucormycosis [26]. Mini-iatrogenic outbreaks have also been documented [27, 28]. Defective ventilation systems and leakage of water have also been associated with outbreaks [9].

Diabetes and hematological malignancies are the major causes of mucormycosis in the developed countries [29], while uncontrolled diabetes, stroke, and trauma are the major underlying factors in developing countries like India [1, 12, 30]. A characteristic finding is that even relatively immunocompetent hosts may land up with serious mucormycosis infection, whereas classically defined immunocompromised hosts (e.g., patients on chemotherapy, transplant recipients, or inherited immunodeficiencies) are affected by other filamentous fungi [18, 31]. As a heterogeneous group of population is affected, defining a specific population at risk in order to target the same is actually difficult. *Mucorales* themselves are ubiquitous in nature, thus posing a serious challenge in monitoring such patients and early detection of the fungus.

4.3.2 COVID and Mucormycosis

A global public health event of concern is the coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [32, 33]. SARS-CoV-2 results in lower respiratory tract infection that may result

in acute respiratory distress syndrome (ARDS) [34]. This outcome is widespread alveolar damage along with serious inflammatory exudation and immunosuppression resulting in reduction of CD4+ and CD8+ T cells [35]. Fungal co-infections are likely to develop in critically ill patients, mainly those in intensive care units (ICUs) who require mechanical ventilation, or those having prolonged duration of hospital stay [35, 36]. Superadded fungal infections may occur during the middle and later stages of the disease, especially in severely ill COVID-19 patients [37]. In the current global scenario with the rampant spread of COVID-19, an issue of concern in India and in some other countries is the occurrence of black fungal infection or mucormycosis in such patients during therapy or even posttreatment as a complication. The incidence of mucormycosis in COVID-19 patients is mainly due to the use of steroids as a mainstay of treatment to suppress highly active immune system. Based on the recent scenario of increasing mucormycosis cases in COVID-19 patients, it becomes important to monitor the patients even after complete recovery from this disease. According to a systemic review report, the common forms of mucormycosis seen in COVID-19 patients are pulmonary mucormycosis, rhino-orbital-cerebral mucormycosis, gastrointestinal mucormycosis, and disseminated mucormycosis [38]. By observing the early symptoms of mucormycosis in such patients, early identification and treatment is possible in order to halt the spread of the disease to other organs as well as to decrease mortality rate [30].

It has been opined that there are different causes for mucormycosis in COVID-19 patients. Incidence of mucormycosis is high in COVID-19 patients with uncontrollable diabetes and those on steroids especially in India, where uncontrolled diabetes mellitus is the most common predisposing factor for mucormycosis [39, 40]. During the infection of COVID-19, the iron containing ferritin level increases in patient's body which acts as a rich source of iron and is a good precursor for the growth of mucormycosis. Another reason is the use of unsterile oxygen. *Mucorales* usually grow in damp areas and can also be found in the inner surface of the water tanks. Hence, it is highly important to examine the water used for oxygenation regularly in COVID-19 patients. Also, the usage of sterile cannula and oxygen mask is highly important to prevent entry of fungal spores in COVID-19 patients [30]. In COVID-19 patients, post organ transplantation, risk for mucormycosis increases as they are on steroids for immunosuppressive activities. In such cases, in spite of antifungals and surgical intervention, the mortality may be very high [41]. A number of cases have been reported till now by doctors across the country. Although steroid usage cannot be stopped in COVID-19 patients, but by taking proper care, by maintaining hygiene, and by early diagnosis and treatment, the spread and severity of mucormycosis can be reduced [30, 42].

Mucormycosis in COVID-19 patients can be prevented by judicious and supervised use of systemic corticosteroids and tocilizumab as per guidelines, continuous monitoring and strict control of diabetes mellitus, following aseptic precautions during oxygen administration, environmental and personal hygiene, oral Betadine gargles, nose and mouth cover by mask, and administration of oral posaconazole prophylactically especially in high-risk patients [43].

4.4 Pathogenesis

An understanding of the pathogenesis of mucormycosis is essential to develop newer strategies for management. Colonization or invasion of deep tissues by *Mucorales* is mainly by inhalation, ingestion, or inoculation percutaneously. The phagocytes of the immunocompetent host are the first line of defense and kill the spores of *Mucorales* mainly by generating oxidative metabolites and cationic peptides called defensins [12, 44–46]. It is due to this feature that severely immunocompromised patients with neutropenia and patients with phagocyte dysfunction (as in hyperglycemia) are at a greater risk of mucormycosis. The spores can invade blood vessels if at all they escape phagocytosis, partly due to effective adherence to endothelial cells. This ability is maintained by *Rhizopus oryzae* even in a nonviable state [47]. The growth of *Mucorales* is inhibited by normal serum. Serum acidosis, in vitro, hence is conducive for the growth of *Rhizopus* spp. [48]. Even though *Mucorales* have been shown to share various immunopathogenic features with other filamentous fungi [49], they possess certain virulence characteristics which are unique, resulting in specific interactions between host and pathogen, which further aid in host evasion and progression of disease making the outcome all the more lethal [50].

Few such characteristics are as follows:

(a) Decreased susceptibility to innate host defense than other commoner fungi [51, 52].
(b) Different patterns of susceptibility to host responses among various species within the order *Mucorales* like triggering of a defective oxidative burst in human phagocytes or an increase in resistance to hyphal damage caused by phagocytes in vitro etc. [53, 54].
(c) Increased virulence owing to production of a stronger pro-inflammatory response [54]. The recognition patterns of the fungal cell wall may be differently recognized by the dectin receptors and toll-like receptors resulting in the modified expression of chemokines and cytokines, i.e., stimulatory molecules for immune response [36, 55].
(d) Widespread angioinvasion resulting in thrombosis of vessels and necrosis of tissues. This causes hematogenous dissemination and ischemic necrosis of the infected tissue, thus preventing the penetration of leukocytes and antifungal agents to the infected areas [50, 56, 57]. An interaction between CotH3 (a spore-coating protein family on the surface of *Rhizopus* spp.) and GRP78 (glucose regulator protein 78 on endothelial surface) has been found to be cause of angioinvasion [58]. CotH3-GRP78 interaction is noted on nasal epithelium also. An interaction between CotH7 and B1 integrin on alveolar epithelial cells activates the EGF signal pathway resulting in invasion of the host cells by the fungus [59]. An increase in the signaling of platelet-derived growth factor receptor B (PDGFRB) occurs when CotH and epithelial cell contact takes place that provides suitable growth factors for the *Mucorales*. CotH3 also plays the role of invasin and mediates immune cell damage, thereby promoting infection. T cells, specific to *Mucorales*, are produced by the mature hyphae which induce

cytokines with pro-inflammatory activity such as interleukins (IL-4, IL-10, and IL-17) and interferon gamma (IFN-γ) that result in stimulation of CD4+ T cells and cause further damage to the host cells. Further, the release of immunomodulatory molecules such as RANTES (regulated upon activation, normal T-cell expressed and secreted) and IFN-γ (produced by natural killer or NK cells) is suppressed by the mature fungal hyphae [60].

(e) Utilization of host iron in patients with diabetic ketoacidosis and patients receiving dialysis or multiple transfusions who are given iron chelator deferoxamine. Deferoxamine is a siderophore that is used by the fungus to get access to iron which was unavailable previously [48, 50, 61]. Due to the acidosis in patients with ketoacidosis, there is dissociation of iron from proteins that trap it, and this in turn enhances the survival and virulence of the fungus. β-Hydroxybutyrate, a ketoacid, boosts host and fungal receptor expression, which promotes fungal adhesion and penetration into tissues [3, 62].

(f) Impairment of ability of macrophages to prevent sporangiospore germination by use of corticosteroids for immunosuppressive therapy and diabetes mellitus [63, 64] resulting in increased pathogenicity. In macrophages, corticosteroids cause impaired migration, ingestion, and phagolysosome fusion. A significant risk factor for mucormycosis is use of high-dose systemic corticosteroids for more than 3 weeks of duration [65]. Phagocytic movement toward the source of infection and lytic capacity by various mechanisms (both oxidative and non-oxidative) is reduced by ketoacidosis [29, 66]. Mucormycosis risk is increased by hyperglycemia even without acidosis, where proteins for sequestering iron are hyperglycated, normal sequestration of iron is disrupted, and GRP78 is upregulated, thus enabling tissue penetration, induction of phagocytic functional defects, and enhanced CotH expression [3].

(g) Recently, it was found that the virulence of *Mucorales* was enhanced selectively after exposure to voriconazole [67]. This was found in hemato-oncological patients in whom voriconazole was given as a broad-spectrum antifungal prophylaxis against *Aspergillus* [68].

(h) Other potentially important virulence factors include *Rhizopus* spp. mycotoxins and lytic enzymes, but their contribution to *Zygomycetes* pathogenicity needs to be evaluated further [50]. Mucoricin is the only toxin identified till now, secreted by *Rhizopus delemar* during spore germination. It resembles the plant toxin ricin and contributes to the pathogenesis by enhancement of angioinvasion, inflammation, and destruction of tissues [69].

4.4.1 Mucormycosis in COVID-19 Cases

Cytokine storm is an unregulated immunological reaction brought on by SARS-CoV-2 infection. Additionally, CD4+ and CD8+ T cells' ability to orchestrate immunological responses in COVID-19 patients declines. Silent hypoxia occurs as a result of the atelectasis due to SARS-COV-2-driven pneumonia. Under normal circumstances, the hypoxia-inducible factor-1 (HIF-1) is dormant, but in COVID-19,

the HIF-1 transcription factor subunit plays a significant role in the activation of innate and adaptive immune cells and hypoxia-induced endothelial cell damage, leading to increased localized inflammation and tissue damage [60].

Two primary cellular reactions are started by the immune system in response to an invasive infection. One is induction of Type 1 interferons, which warns the neighboring cells and prepares them to ward off the virus. Another response is the activation of the transcription factor nuclear factor kappa B (NF-κB), which promotes the release of chemoattractant proteins that draw cells of the innate and adaptive immune system, such as T cells, B cells, monocytes, neutrophils, and natural killer (NK) cells, to the site of infection. SARS-CoV-2 is distinctive in that it inhibits INF-1 while inducing NF-κB, enabling unrestricted SARS-CoV-2 replication. Hence, the amount of immune infiltrate generated is significant with neutrophil infiltration being most dominant ultimately causing cytokine storm. Respiratory distress occurs due to the infiltrate in combination with a blocked interferon induction. Fungal invasion is more likely due to direct airway epithelial cell injury and inhibition of INF-1 pathways. A risk factor for mucormycosis is further use of corticosteroids. Altered iron metabolism is seen in COVID-19 infections in the form of hyperferritinemic syndrome [60].

4.5 Clinical Manifestations

Sinuses are most involved in mucormycosis (39%), followed by the lungs (24%), skin (19%), brain (9%), gastrointestinal tract (7%), and other sites (6%), or it may manifest as a disseminated illness (6%) [18]. Most cases (44–49%) of mucormycosis are of the rhino-orbito-cerebral type, with cutaneous (10–16%), pulmonary (10–11%), disseminated (6–11.6%), and gastrointestinal (2–11%) presentations following [70–72]. The clinical diagnosis of mucormycosis, except for rhino-cerebral and cutaneous mucormycosis, is challenging and frequently made at a later stage of the disease or at post-mortem [73]. After infection, black-colored lesion is produced due to which mucormycosis is also known as black fungus [30].

Rhino-cerebral form accounts for majority (33–50%) of all mucormycosis cases. Uncontrolled diabetes mellitus and leukemia are the most common underlying conditions linked to this clinical presentation [2, 4, 25, 74–77]. Necrosis of the palate or sinuses may be the first clinical sign that may advance to the orbit before affecting the intracranial tissues and causing cavernous sinus thrombosis and cranial invasion [3, 78, 79]. Black sores on the palate or nasal mucosa, together with clinical association, are particularly suggestive of mucormycosis, albeit they may not be present in 50% of cases [80, 81]. This form results in very high mortality ranging from 30% to 69%. A bad prognosis is indicated by treatment delays of more than 6 days, invasion of intracranial structures or palate, and bilateral involvement and in case of hematological malignancies [25, 82].

The next frequent manifestation is invasion of the lung following the inhalation of spores resulting in respiratory manifestations with a mortality rate of over 60% [25]. It is challenging to distinguish between mucormycosis and invasive

aspergillosis as the most frequent underlying diseases observed are neutropenia-associated hematological malignancy and diabetic mellitus [4, 73]. Symptoms are nonspecific and include fever, hemoptysis, and pleural discomfort. Tracheal or endobronchial lesions are frequent [83, 84], and large vessel involvement may result in fatal hemoptysis [85]. Invasive aspergillosis and mucormycosis both exhibit radiological features of vascular invasion, thrombosis, and tissue necrosis, which creates a difficult diagnostic situation.

Central nervous system involvement may occur as the disease progresses from the rhino-orbital pathway or as a central nervous system-specific clinical manifestation [86, 87]. Intravenous drug use may predispose to mucormycosis of the central nervous system [88, 89]. Magnetic resonance imaging scans frequently show lesions and basal lymph node involvement. Cerebrospinal fluid may exhibit nonspecific meningitis signs, although yield in culture is quite low [25].

Cutaneous and soft tissue mucormycosis can develop on healthy skin or after a barrier has been compromised, such as after surgery, trauma, burns, catheter insertion sites, or even bug bites. Additionally, it has been reported following intramuscular injections and the use of contaminated dressings [25]. Pustules, vesicles, or wounds with large necrotic zones are some of the clinical signs of cutaneous mucormycosis. Lesions may resemble ecthyma gangrenosum lesions in their early stages. The death rate for necrotizing fasciitis brought on by mucormycosis is close to 80% [3].

Involvement of the digestive tract may also occur with up to 98% mortality [90]. Delivery of medications prepared using applicator sticks that may be contaminated may result in nosocomial gastrointestinal illness [3]. The vague features of gastrointestinal mucormycosis, such as hematemesis or melena or just abdominal pain, result in delayed diagnosis [91].

Mucormycosis may cause endocarditis in natural or prosthetic valves and may result in intrusion and blockage of the major vessels with very high mortality. Nephrectomy is a necessary component of treatment when mucormycosis affects the kidneys. Certain occurrences of peritoneal mucormycosis have been reported in patients receiving continuous ambulatory peritoneal dialysis, which typically develop as a sequela of an old bacterial peritonitis [25]. Mucormycosis may rarely present as osteomyelitis which may be secondary to other forms of mucormycosis or by inoculation following trauma or surgery [2]. Leukemia patients and intravenous drug users may show brain involvement (usually the basal ganglia) without rhino-orbital involvement [92]. Disease involving only the trachea, ear, mastoid, bone, oral mucosa, bladder, or mediastinum alone is uncommon [12].

Involvement of more than two contiguous organs is labeled as disseminated mucormycosis. 23–62% of such cases are seen in patients having hematological disorder with severe immunosuppression and neutropenia [93, 94]. Corticosteroid use, immunosuppression post organ transplant, chemotherapy, and deferoxamine use are risk factors. Itraconazole or voriconazole prophylaxis may also predispose to disseminated disease [3]. Mortality of nearly 100% has been associated with this form [2, 94].

The varied presentations usually make early identification and management difficult.

4.6 Diagnosis

Accurate and early diagnosis is the mainstay for improving the prognosis of mucormycosis. But most cases are confirmed only at postmortem evaluation [6, 95, 96]. The nonspecific clinical appearance of mucormycosis and many shortcomings of the currently used diagnostic methods are factors that make a diagnosis difficult. In contrast to renal (44%), pulmonary (31%), disseminated (0%), and gastrointestinal (0%) forms of mucormycosis, Chakrabarti et al. found that cutaneous (100%) and rhino-orbito-cerebral (91%) mucormycosis were most accurately detected antemortem [73].

Certain clinical manifestations like necrotic naso-sinus eschars, diplopia, necrotic cutaneous lesions, pleuritic pain, etc. have a high predictive value [12, 96] but they are nonspecific. Infections by other pathogens with angioinvasive nature, such as *Aspergillus* spp., *Scedosporium* spp., *Fusarium* spp., and *Pseudomonas aeruginosa*, are considered as differential diagnosis. Early detection of mucormycosis necessitates a suspicion of high index and timely identification of host risk variables. *Mucorales* are ubiquitous, and hence invasive mucormycosis can be definitively diagnosed by histopathology studies or a positive sterile site culture (such as a needle aspirates, tissue biopsy material, or pleural fluid) [3].

The current modalities available for diagnosis are as follows:

(a) **Direct examination:** Direct microscopy can be accomplished using a variety of methods, including 20% potassium hydroxide treatment, Gomori's methenamine silver staining, hematoxylin and eosin staining, and periodic acid-Schiff staining. A quick presumptive diagnosis is possible with the use of optical brighteners in clinical specimens, such as Blankophor and Calcofluor White [97]. *Mucorales* have broad, ribbonlike aseptate to pauciseptate hyphae with hyphal branching angles ranging from 45 to 90 degrees, according to traditional descriptions [98]. But wet mounts may not suffice in detection of hyphae of *Mucorales* or hyphae may not be abundant enough to be seen.

(b) **Fungal culture and identification:** This is important for epidemiology, prognosis, and therapy [1]. *Mucorales* can be grown on routine culture media for bacteria and fungi, like Sabouraud's dextrose agar with 25–55 °C being the growth temperature range. *Mucorales* found in clinical specimens grow at 37 °C, forming cottony white, gray, or brownish colonies that quickly fill the Petri dish, usually within 24–96 h (latest by 1–7 days) [4, 71, 99]. *Mucorales*' recovery from clinical microbiology laboratory specimens is difficult. Invasive procedures to obtain the required material may pose a significant risk, particularly in critically ill patients with coagulopathy or thrombocytopenia. Culture has limited sensitivity as homogenization or grinding the specimen aggressively before plating may destroy the coenocytic hyphae [1, 2, 25]. Preferable is to cut

such specimen into small pieces prior to plating. As optimal growth temperature varies greatly between isolates, it is recommended to incubate cultures at room temperature and 37 °C [3].

R. pusillus and *Lichtheimia corymbifera* have been successfully identified using the ID32C kit (bioMérieux, Marcy-l'Étoile, France). The API 50CH (bioMérieux) [100] has been used for identifying *Mucor* species. However, neither test could differentiate *M. circinelloides* and *M. rouxii*. ID32C along with assimilation of melezitose detects *L. ramosa* [101]. Wide, aseptate hyphae on slides or in culture should always be viewed cautiously because they could represent colonization. Confirmation needs a combination of symptoms that are consistent with tissue invasion as shown histologically.

(c) **Histopathology:** The most accurate diagnosis can still be made through biopsy and histopathologic analysis. Microscopically, distinctively wide (between 6 and 30 μm), thick-walled, ribbonlike aseptate hyphal components with right-angle branches can be detected. Other fungi have septa that are thinner and branch at acute angles, including *Aspergillus*, *Fusarium*, and *Scedosporium* species. Sometimes when tissue is processed, folding causes artificial septa to form. Hematoxylin and eosin or periodic acid-Schiff stains are best for visualizing the *Mucorales* [3]. Cultures are negative in two-thirds of cases that are diagnosed histopathologically [102]. HPE also aids in differentiating whether the fungus is a pathogen or culture contaminant. Angioinvasion and infarction of surrounding tissue are common findings. Perineural invasion was noted by Frater et al. in 90% of biopsies, which may explain the occurrence of rhino-orbital-cerebral disease [98, 103]. It is essential to explain the histological vessel invasion, by aseptate, wide hyphae branching at right angles because this is diagnostic in the right clinical context [25, 104]. In their analysis of 20 cases, Frater et al. found that a neutrophilic inflammatory response was present in 50% of cases, neutrophils and granulomas together were present in 25% of cases, granulomas alone were seen in 5% of cases, and inflammatory response was absent in 20% of cases. This suggests that the inflammatory response produced by *Mucorales* may be variable [98] which may pose a problem in accurate diagnosis. The main drawback of histopathological testing is that the genus and species are not identified, therefore requiring cultural correlation.

(d) **Biomarkers/serology:** *Mucorales* lack 1,3-β-D-glucan (BDG) and galactomannan (GM) in their cell walls. Therefore, for diagnosis of mucormycosis, neither of the biomarker assays are helpful [105]. But they may be of negative predictive value as BDG is negative in mucormycosis (97.8% specificity) and GM is usually negative in mucormycosis (89% specificity) [56, 106].

When cultures are negative, immunohistochemistry utilizing monoclonal antibodies against *Rhizopus arrhizus* (recently made available commercially) can help with the diagnosis. It has been shown to have 100% sensitivity and specificity for distinguishing aspergillosis from mucormycosis [107, 108] and has been recommended by European Confederation of Medical Mycology [106].

(e) **Molecular methods:** Molecular identification is possible, which supports rapid and precise diagnosis. The most reliable method for molecular systematics at the species level has been ITS sequencing, which is advised as the first-line test for *Mucorales* species identification [106]. In some cases, the only material available for diagnosis is biopsy samples which are formalin-fixed and paraffin-embedded, and these can also be used for molecular tests. In these situations, most molecular methods rely on PCR assays, which may perform poorly since formalin fixation is linked to DNA damage. Further drawbacks of molecular methods are capacity for providing quick results, time to detection, and limited sensitivity [96, 109].

Another tool having a high accuracy for determination of fungi from cultures is MALDI-TOF [110]. Vitek MS (France) and Bruker Biotyper (Germany) are the currently commercially available FDA-approved MALDI-TOF MS platforms. The benefit of this method is that it quickly identifies fungi in isolates or direct clinical specimens within 30 min. T2 magnetic resonance (T2MR) (T2 Biosystems) is another commercially available FDA-approved platform where the complex of target organism and target-specific agent-coated particles is observed in the altered microenvironment of the surrounding water molecules, aiding in quick identification even when levels of fungus is low [60]. Also, for detection of invasive mold infections from blood specimens, next-generation sequencing (NGS) may be useful [111]. For detection of fungi in tissues, several methods have been developed such as nested PCR, real-time PCR, nested PCR with RFLP [112], PCR with electrospray ionization mass spectrometry (PCR/ESI-MS) [113], and PCR with high-resolution melt analysis (HRMA) [114]. Many of these techniques, which work better on fresh or deeply frozen samples than on paraffin-embedded tissues, have apparently been effectively used. The success of the PCR depends on the choice of target [115]. The targets evaluated so far are 18S rRNA, 28S rDNA, iron permease I gene FTRI or cytochrome b, the mitochondrial gene *rnl*, or the specific CotH gene that aid in identification of *Zygomycetes* species from cultures [9, 116].

When tested on serial blood samples from patients with culture-positive invasive mucormycosis, a new commercial pan-*Mucorales* real-time (qRCR) kit (MucorGenius®, PathoNostics, Maastricht, the Netherlands) was found to be a quick user-friendly diagnostic test with 75% overall sensitivity and provided results a few days to weeks prior to final diagnosis. Genus identification is not feasible, and it might not be able to detect a light fungal burden [117, 118].

In samples, where histopathology is suggestive of characteristic *Mucorales*, molecular methods usually confirm the diagnosis and thus are recommended [106]. As per recent studies, qPCR on BAL samples has been found to be of value with respect to quicker diagnosis and better prognosis in pulmonary mucormycosis cases [114].

(f) **Imaging:** In immunocompromised patients with invasive disease, diagnostic imaging, particularly CT scan, is important for early diagnosis. Simple radiography and computed tomography studies in rhino-orbital disease reveal sinus, orbit displacement, and invasion of the surrounding bone structures. Magnetic

resonance imaging is the preferred method when intracranial structures are impacted [25]. But features seen radiologically are not characteristic of mucormycosis and infection cannot be excluded by their absence. Numerous pulmonary mucormycosis-related lesions, including nodules, cavities, halo signs, wedge-shaped lesions, reverse halo signs, and pleural effusions, may also be seen in other angioinvasive organisms, such as *Aspergillus* spp., *Scedosporium* spp., *Fusarium* spp., and *P. Aeruginosa* [96, 119]. PET CT using [18F]-fluorodeoxyglucose (FDG) can also aid in the detection and treatment of mucormycosis [120]. When possible, endobronchial fine needle aspiration under ultrasound guidance is also a useful diagnostic tool [121].

Future diagnostic prospects:

- **Molecular**: In spite of the *Mucorales* being angioinvasive, blood cultures remain negative. But fungal DNA may still circulate in the blood. As a result, there is a lot of focus on noninvasive techniques (like qPCR) for the detection of mucoralean DNA that is present in the urine or blood. Multiple studies have demonstrated that serum *Mucorales* PCR is a highly accurate and quick diagnostic tool with low detection threshold for the diagnosis of invasive mucormycosis in immunocompromised patients, detecting the infection 3–68 days sooner than the standard approaches. This method may also assist in monitoring of therapy [9]. Techniques such as WGS and CRISPR-Cas9 that detect the cell-free DNA (cfDNA) in blood may be useful as an aid to early diagnosis owing to their high sensitivity.
- **Serology**: New serological targets are being researched, and this is challenging as no biomarkers, specific for mucormycosis, are available for rapid identification. A sandwich ELISA using monoclonal antibody (2DA6) has been evaluated and has been shown to react strongly with the purified fucomannan of *Mucor* spp. The next test evaluated on clinical samples was a lateral flow immunoassay (LFIA) that detected fucomannan present in cell walls of *Mucorales*. LFIA could be used as a rapid test on serum, BAL, tissue, and urine and was easier to use than ELISA. In mouse models, within 3–4 days of infection, the test was able to quickly and precisely identify *R. delemar*, *L. corymbifera*, *M. circinelloides*, and *C. bertholletiae* [9]. Techniques including ELISA, immunohistochemistry, immunodiffusion, and immunoblots could be further examined for commercial application on the basis of the specific antigens [60]. Potentially useful as a reliable marker for the detection of invasive mucormycosis are *Mucorales*-specific T lymphocytes [122].
- **Metabolomics (breath test)**: *Rhizopus arrhizus* var. *arrhizus*, *R. arrhizus* var. *delemar*, and *R. Microsporus*, the three species which commonly affect human, were used to study the breath profiles of volatile metabolites in an experimental invasive mucormycosis mouse model. The method used was thermal desorption-gas chromatography-tandem mass spectrometry (GC-MS), and controls used were *Aspergillus fumigatus*-infected mice. It was seen that the above *Mucorales* species exhibited different breath profiles of sesquiterpene, a volatile metabolite. This could aid in identification of such in vivo infections along with

differentiating them from each other and also from aspergillosis. Thus, this is a potential noninvasive method that can be used for diagnosis and also for monitoring the therapeutic response. Moreover, it may be used as an additional diagnostic tool along with *Aspergillus* galactomannan, in high-risk patients like those with neutropenia post leukemia treatment or those being taken up for stem cell transplantation, to screen for mold infections. But even though promising, it needs further evaluation [123].

– **Biosensors:** Technologies employing biosensors have been found to be useful for the detection of fungi. Biosensor is an analytical tool that converts physical or chemical signals into electrochemical or optical signals in order to measure the concentration of a bio-analyte. These have been experimented in case of *C. albicans* and *Aspergillus fumigatus*. The development of biosensors would be of utmost importance given the current global situation, for the quick and early identification of mucormycosis, as well as for monitoring response to treatment based on particular marker analytes.

Accurate and rapid diagnosis of mucormycosis is very important, and diagnosis is based on quick risk factor recognition, clinical findings, and radiological features, along with detection of fungus in clinical specimen, by direct (culture, microscopy, and histopathology) or indirect (antigen detection or identifying cell wall constituents) methods until newer methods of diagnosis (molecular and biomarkers) are developed and approved [25, 30, 96]. In the future, the availability of cutting-edge, minimally intrusive techniques for safe sampling of tissues and the detection of pathogens by molecular methods may clarify many murky clinical situations [124].

4.7 Management

The management of mucormycosis consists of rectification of underlying factors, debridement or resection of the affected tissue, and adequate therapy with antifungal agents. Diagnostic delay may worsen the prognosis and hence high suspicion index is necessary [25]. Diabetic ketoacidosis and corticosteroids/deferoxamine suppression are predisposing factors that can be managed. The prognosis of invasive fungal infections in the immunocompromised has improved overall owing to the use of new antifungal drugs. However, the mortality documented overall is more than 50% as the *Mucorales* are invasive in nature and many a times use of antifungal therapy alone is ineffective resulting in 100% mortality mainly in patients having disseminated infection [6, 18]. In mucormycosis, thrombosis of blood vessels and the ensuing tissue necrosis might make it difficult for antifungal medicines to reach the infection site. Necrotic tissue debridement is therefore essential for the death of illness. If possible, early and proper surgery is the best for mucormycosis therapy. In very extended forms, surgery plus antifungal medication is always preferable to antifungal therapy alone [25]. A delay in treatment (amphotericin B therapy) of more than 6 days of initial infection increases the mortality rate by two folds at the end of 12 weeks post the diagnosis [125].

The absence of significant clinical studies is one of the major challenges in researching the therapy of mucormycosis. The disease's rarity, high associated costs, challenge of designing multicentric trials, and existence of variables, such as surgery, that might impede the interpretation of data, are a few possible explanations for the same. The results of antifungal susceptibility testing studies, the findings of studies using animal models, and the knowledge obtained from human cases all play a role in the selection of the best antifungal agent [25]. The pharmacological target for treatment of fungal diseases becomes limited because target sites of eukaryotic pathogens are similar to those in humans [126].

By substituting just one amino acid in the sequence alignment of the azole target – lanosterol 14a-demethylase CYP51 F5 proteins—*Mucorales* exhibit intrinsic resistance to short-tailed azoles. The overexpression of multidrug transporters is another frequent method of acquired azole resistance. Transient resistance has also been reported as a result of an acquired epigenetic mechanism. In the drug's presence, this technique silences the gene responsible for encoding the target protein or the enzymes that transform the drug into a harmful chemical, thus producing epimutants that are RNA interference (RNAi)-dependent [59]. Different *Mucorales* react differently to antifungal medications. For instance, *Mucor circinelloides* exhibits greater posaconazole susceptibility, *Rhizopus oryzae* exhibits in vitro posaconazole resistance, and *Cunninghamella* often has higher MICs for amphotericin B [96]. Additionally, the same species may respond differently to the same antifungal group. For instance, *Rhizopus oryzae* responds differently to various triazoles, with voriconazole having less activity than posaconazole, possibly as a result of divergence and increased copy number of the triazole target gene, i.e., duplicated ERG11 [127]. As a result, *Rhizopus oryzae* exhibits four to eight dilutions higher MICs for itraconazole and posaconazole than those for *Aspergillus fumigatus*, and fungicidal activity is not attained over a safely feasible range of drug concentrations. Patients on voriconazole frequently develop *Rhizopus oryzae* infections suggesting that this antifungal medication is inefficient against *Rhizopus oryzae*.

Targets Available for Mucormycosis Therapy These include (1) binding of polyene to cell membrane ergosterol, causing pore formation in the membrane; (2) cytochrome p450 enzyme (14-α-demethylase) inhibition by posaconazole, resulting in disruption of ergosterol synthesis; (3) interference with fungal cell wall β-glucan cross-linking by echinocandin; and (4) iron chelation therapy with deferasirox that blocks iron uptake essential for growth. Additionally, adjuvant therapy using host immune boosting techniques is possible and includes (5) transfusion of granulocytes and (6) cytokine-based therapy [128].

(a) **Antifungal agents** [106, 129]**:** Amphotericin B (polyene antifungal) is treated as the mainstay of therapy for mucormycosis and should generally be included in primary treatment. Amphotericin B exhibits variable in vitro activity against causative agents of mucormycosis. Lipid formulations of amphotericin B are less toxic and thus are tolerated better than amphotericin B deoxycholate [25]. Based on retrospective survival data and better penetration of brain tissue,

liposomal amphotericin B (LAmB) is preferred over amphotericin B lipid complex (ABLC) for the therapy of central nervous system (CNS) infection [3]. The optimum dosage and time for starting treatment at each infection site, however, are the key concerns. A retrospective study by Chamilos et al. determined the effect of prompt amphotericin B-based treatment on the outcome of patients with mucormycosis. They noticed a twofold increase in mortality in patients having both hematological malignancy and mucormycosis as a result of delayed amphotericin B-based therapy as compared to early treatment [125].
Triazoles are the next commonly used agents mainly as salvage therapy. Both posaconazole and isavuconazole are FDA approved for induction and salvage therapy [30]. Posaconazole and ravuconazole have been shown to have effective activity in vitro. Posaconazole has outperformed itraconazole in animal models, but it is less efficacious than amphotericin B deoxycholate [25]. Recent research shows that posaconazole can be used compassionately when other medicines, including amphotericin B, have failed to treat patients' condition [130, 131]. It is presently the most potential therapeutic substitute for amphotericin B. Mucormycosis has been successfully treated with liposomal amphotericin B as the initial treatment, followed by posaconazole as a follow-up [30]. Experts believe that lipid polyenes are the first-line treatments, and like posaconazole, isavuconazole is best saved for step-down oral therapy in patients who have improved on polyene-based therapy or for salvage therapy in patients intolerant to polyene-based regimens or whose infection is resistant to these regimens [3]. In spite of its activity against *Mucorales* in vitro, itraconazole is deemed inappropriate for therapy and leads to treatment failures [130, 131]. Voriconazole is ineffective against *Mucorales* both in vitro and in vivo [25]. Therapeutic monitoring is needed while using triazoles for treatment of mucormycosis to prevent unwanted adverse effects [30].

Echinocandins have almost no activity against *Mucorales* in vitro; however, they do have a small amount of activity in vivo [132]. Even though caspofungin has low in vitro action against the pathogens causing mucormycosis, an in vitro versus in vivo link has not been fully established. Low-dose caspofungin increased the survival of mice with disseminated mucormycosis caused by *Rhizopus oryzae* that was predisposed by diabetic ketoacidosis in an experimental model and demonstrated activity in vitro by inhibiting the (1,3)-b-D-glucan synthase of *Rhizopus oryzae*. Given the possibility of a synergy between caspofungin and the amphotericin B lipid complex, additional research may be required to determine caspofungin's role in mucormycosis treatment [25].

The use of polyene-azole combination regimens rather than polyene monotherapy or polyene-echinocandin combination regimens is not backed by sufficient data. For individuals with severe disease or whose condition has worsened despite treatment, some specialists recommend triple therapy, which includes a polyene, an echinocandin, and either posaconazole or isavuconazole. When both septate molds and mucormycosis are considered as differential diagnosis and the causative agent has not been identified yet, empirical dual lipid polyene-azole therapy seems to be a sensible option in a patient with

potentially invasive mold infections [3]. Although there was no difference in the tissue fungal load, the combination increased survival rates in an experimental mouse model of disseminated mucormycosis, when compared to each drug used individually [133]. For those patients who were intolerant to amphotericin B, the combined regimen of posaconazole with caspofungin proved to be effective probably due to the potential synergistic effects between two drugs.

(b) **Chelating agents:** Another therapeutic alternative is usage of iron chelants apart from deferoxamine, although its efficacy needs further evaluation. Deferasirox, an iron chelator, has shown to have fungicidal activity against clinical isolates of the *Mucorales*. Combined deferasirox-LAmB therapy improved survival rates in mice with disseminated mucormycosis and diabetic ketoacidosis while also lowering the amount of fungi in the brain. Unfortunately, a phase 2 clinical trial that was randomized and double-blind, in which deferasirox was used as adjunctive therapy with LAmB, showed a higher mortality in patients on deferasirox treatment. The study population consisted of patients' malignancy having active disease, and only few with the risk factor of diabetes mellitus. Deferasirox is therefore not recommended as a treatment for individuals with active cancer, but its role in those with diabetes mellitus without active cancer (the situation in which its preclinical efficacy was greatest) is unclear [3].

(c) **Hyperbaric oxygen therapy:** This is an alternative that needs more research, particularly in patients with the rhino-cerebral variant of disease [134, 135]. Although its effectiveness is yet unknown, there is very little evidence to justify the use of hyperbaric oxygen in facilities with the necessary technical know-how and resources [3].

(d) **Immunomodulators:** Granulocyte-macrophage colony-stimulating factor and interferon gamma are cytokines that have also been used for mucormycosis treatment [136, 137]. Although it is well established that earlier neutrophil level recovery increases survival chances, the roles of recombinant cytokines and neutrophil infusions in the initial treatment of mucormycosis are unclear [3]. Granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interferon-γ (IFN-γ) are recombinant cytokines that can activate granulocytes resulting in fungal damage. Transfusion of granulocytes can also be used to transfer polymorphonuclear leukocytes to the infection site in hosts with neutropenia. Amphotericin B in lipid form and polymorphonuclear leukocytes are synergistic and harm *Rhizopus* species' hyphae. A case study article showed that an immunocompetent patient who was not responding to conventional therapy has benefited from the combination of nivolumab and interferon gamma [138].

The antifungal treatment for mucormycosis needs to be given until the underlying immunosuppression and clinical signs and symptoms of infection have resolved. In a patient who is clinically improving, as maintenance therapy, one might move to taking lipid polyene doses three times weekly before reducing them to twice weekly. Antifungal prophylaxis is continued as long as the immunosuppressive regimen is used, in patients being given immunosuppressants. The importance of radiographic

follow-up is a typical problem in long-term management, and more attention should be paid to clinical response, especially during first 2–4 weeks following the start of therapy [3].

4.7.1 Future Treatment Modalities under Research

VT-1161 A drug under evaluation, this is an inhibitor that has been shown to have in vitro efficacy against *Mucorales*, including *R. oryzae*, *Lichtheimia*, and *Cunninghamella* by exerting selective activity against the fungus CYP51. Neutropenic mice infected with *R. oryzae* have showed that VT-1161, when administered either prophylactically or therapeutically, increases overall survival. Although further research is needed to see whether VT-1161 is beneficial against other *Mucorales*, this ergosterol production inhibitor may be effective against mucormycosis [139].

Immunotherapies Research is now being done on immunotherapies that are effective against mucoricin and CotH proteins which are fungal ligands. These treatments, along with others, must be developed because investigational antifungals that are now being tested only have weak action against *Mucorales* [59].

Biosynthesized Silver Nanoparticles (AgNPs) AgNPs are frequently utilized to treat conditions including aspergillosis and candidiasis. Several mechanisms, including disintegration of cell wall, degradation of surface protein, damage of nucleic acid by the creation and accumulation of reactive nitrogen and oxygen species, and proton pump blockage, may be responsible for antimicrobial effectiveness of AgNPs against microorganisms. The electron transport system is hypothesized to be harmed by the accumulation of silver ions brought on by AgNPs, which prevents respiration by generating intracellular ion efflux. Numerous studies have been carried out to assess the efficacies of such AgNPs. A recent study showed that when evaluated against *Mucor racemosus*, *Rhizopus microsporus*, and *Syncephalastrum racemosum*, the AgNPs prepared from soil bacterium *Pseudomonas indica* were found to have potent antifungal and antioxidant properties with minimal cytotoxicity in in vitro cell lines [140].

Antiviral Polymerase Inhibitors In a study, it was discovered that eight modified GTP compounds, the drugs remdesivir and sofosbuvir, and the nucleotides, namely, adenosine triphosphate (ATP), guanosine triphosphate (GTP), uridine triphosphate (UTP), and cytosine triphosphate (CTP), all demonstrated similar anticipated binding affinity toward RdRp of *Rhizopus oryzae*. As a result, the study concluded that antiviral medications (anti-RdRp) would be an effective treatment for mucormycosis and may inhibit both *R. oryzae* RdRp and SARS-CoV-2 due to dual antifungal and antiviral activities. However, to demonstrate this theory, additional experimental validations are required [141].

Probiotics They are known to have antifungal and antimycotoxigenic properties in addition to their anti-inflammatory properties. Their role has been studied in case of infections due to *Aspergillus* spp., *Fusarium* spp., *Candida* spp., and others. Future research could focus on evaluating the effectiveness of probiotics alone or as a supplement to current management strategies in the treatment of mucormycosis, particularly when COVID-19 is an associated entity, where they may be used as an anti-inflammatory agent, as a gut dysbiosis restorer, and as an antimicrobial agent [60].

Plant Derivatives Plant-based natural substances are increasingly being used as primary molecules to explore new treatment possibilities. It has been demonstrated that phytomolecules contain antifungal characteristics that work against several fungal species. Based on their binding affinities to the *Rhizopus oryzae* polyprotein (RVT_1 region), Banik et al. attempted to assess various plant-derived antifungal metabolites as inhibitory agents of *R. oryzae* in their investigation. Emetine, jatrorrhizine, isoboldine, and 6-a-hydroxymedicarpin were the four metabolites that were investigated and demonstrated the highest binding affinity with the targeted polyprotein (RVT_1 region), after which their drug profiles were examined using an ADME (absorption, distribution, metabolism, and excretion) analysis. Numerous investigations have suggested that emetine and jatrorrhizine have COVID-19 inhibiting efficacy. Therefore, more in vivo studies are required to confirm these findings [142].

Mucorales-specific inhibitory effects of endophytic fungi are being assessed for their antifungal efficacy. Healthy *Moringa oleifera* leaves were used to isolate endophytic *Aspergillus terreus*. On GC-MS, fungal extract was found to contain 16 major bioactive compounds resulting in promising antifungal activity against *Rhizopus oryzae*, *Mucor racemosus*, and *Syncephalastrum racemosum*, warranting further evaluation [143].

4.8 Clinical and Research Issues to be Addressed for Future Prospects [144]

Epidemiological issues:

- Prospective global registries to be maintained for assessing the prevalence of mucormycosis, efficacy of antifungal treatment, and the identification of vulnerable host populations.
- Molecular fingerprinting techniques to be developed to distinguish between nosocomial and community-acquired types of mucormycosis.

Pathogenetic issues:

- Comparative research to be done on the immunopathogenesis of aspergillosis and mucormycosis.

- Identification of defects in the innate immunity of the host that increase risk.
- Availability of innovative animal models in phylogenetically different hosts.
- Molecular tools to be developed for analyzing molecular Koch's postulates pertaining to virulence of *Zygomycetes.*

Diagnostic issues:

- Availability of antigen-based or molecular detection techniques for rapid diagnosis.
- Clarity regarding radiologic and host factors for mucormycosis diagnosis when compared to other mold infections.

Clinical research-related issues:

- Formulation of guidelines to decrease any deviation from prescribed practice.
- Lack of creative research designs.
- To use risk stratification and define comparators for monotherapy or monotherapy vs combination therapy trials.
- Defining the full transcriptome for colonization and adhesion to epithelia so as to identify new targets for intervention.
- Identification of genetic expression patterns during growth of hyphae and invasion of tissues, as future targets.
- Identification of various factors in blood that can limit the growth of *Rhizopus* spp. and halt further dissemination.
- Identification of signaling pathways used by *Zygomycetes* to escape from the endothelial lining blood compartments leading to growth in deep organs.

4.9 Conclusions

A developing fungal infection with a high fatality rate is mucormycosis. Patients with hematological malignancies and stem cell transplant recipients who are often exposed to voriconazole appear to be at higher risk of developing mucormycosis. An increase in cases has also been noted in the current COVID-19 pandemic situation even though diabetes remains the most common underlying factor globally. For a better knowledge and assessment of the infection, more studies on particular patient populations are required, such as leukemia patients, transplant recipients, and diabetics. Clinicians are challenged by the numerous clinical signs and symptoms and evasive nature of mucormycosis, which frequently causes delays in diagnosis and results in poor prognosis. Given the high mortality rate of this lethal infection, a high index of suspicion based on predisposing risk factors and enhanced laboratory diagnosis are prerequisites. Therefore, new methods need to be developed for rapid detection of mucormycosis. The diagnosis of mucormycosis would be greatly aided by the use of sophisticated molecular techniques such as GC-MS/MS, MALDI-TOF, and T2MR in conjunction with culture-based approaches. To solve the problem of

fast detection of *Mucorales*, new biosensor platforms might also be created by fusing certain molecular biomarkers or recognition components that are specific. It would also be essential for the development of implantable or microchip-based biosensors for continuous fungal analyte monitoring throughout therapy to emphasize the vital points during therapeutic intervention. Amphotericin B and surgical debridement are the first-line treatments; additional antifungal medications might also be utilized. Hence, to prevent these infections and enhance their treatment, it is crucial to manage and control underlying disorders.

References

1. Katragkou A, Walsh TJ, Roilides E (2014) Why is mucormycosis more difficult to cure than more common mycoses? Clin Microbiol Infect 20(Suppl. 6):74–81
2. Prabhu RM, Patel R (2004) Mucormycosis and entomophthoramycosis: a review of the clinical manifestations, diagnosis and treatment. Clin Microbiol Infect 10(suppl 1):31–47
3. Jameson JL, Fauci AS, Kasper DL, Hauser SL, Longo DL, Loscalzo J (eds.) (2020) Mucormycosis. In Harrison's principles of internal medicine, 20th ed., McGraw Hill Education, USA, pp.1537–1541
4. Ribes JA, Vanover-Sams CL, Baker DJ (2000) Zygomycetes in human disease. Clin Microbiol Rev 13:236–301
5. Lanternier F, Dannaoui E et al (2012) A global analysis of mucormycosis in France: the RetroZygo study (2005–2007). Clin Infect Dis 54:S35–S43
6. Skiada A, Pagano L et al (2011) Zygomycosis in Europe: analysis of 230 cases accrued by the registry of the European Confederation of Medical Mycology (ECMM) working group on zygomycosis between 2005 and 2007. Clin Microbiol Infect 17:1859–1867
7. Chakrabarti A, Das A et al (2006) The rising trend of invasive zygomycosis in patients with uncontrolled diabetes mellitus. Med Mycol 44:335–342
8. Chakrabarti A, Singh R (2014) Mucormycosis in India: unique features. Mycoses 57(Suppl. 3):85–90
9. Skiada A, Pavleas I, Drogari-Apiranthitou M (2020) Epidemiology and diagnosis of mucormycosis: an update. J Fungi 6:265–285
10. Troiano G, Nante N (2022) Mucormycosis—emerging fungal threats. Encyclopedia 2:247–255
11. Ruping MJ, Heinz WJ et al (2010) Forty-one recent cases of invasive zygomycosis from a global clinical registry. J Antimicrob Chemother 65:296–302
12. Petrikkos G, Skiada A et al (2012) Epidemiology and clinical manifestations of mucormycosis. Clin Infect Dis 54(suppl 1):S23–S34
13. Bitar D, Van Cauteren D et al (2009) Increasing incidence of zygomycosis (mucormycosis), France, 1997–2006. Emerg Infect Dis 15:1395–1401
14. Chkhotua A, Yussim A et al (2001) Mucormycosis of the renal allograft: case report and review of the literature. Transpl Int 14:438–441
15. Park BJ, Kontoyiannis DP et al. (2004) Comparison of mucormycosis and fusariosis to invasive aspergillosis (IA) among transplant recipients reporting to TRANSNET. In: program and abstracts of the 44th Interscience Conference on Antimicrobial Agents and Chemotherapy (Washington, DC). Washington, DC: American Society for Microbiology
16. Singh N, Aguado JM et al (2009) Mucormycosis in solid organ transplant recipients: a prospective, matched case-control study to assess risks for disease and outcome. J Infect Dis 200:1002–1011

17. Singh N, Gayowski T et al (1995) Invasive gastrointestinal zygomycosis in a liver transplant recipient: case report and review of zygomycosis in solid-organ transplant recipients. Clin Infect Dis 20:617–620
18. Roden MM, Zaoutis TE et al (2005) Epidemiology and outcome of zygomycosis: a review of 929 reported cases. Clin Infect Dis 41:634–653
19. Daly AL, Velazquez LA et al (1989) Mucormycosis: association with deferoxamine therapy. Am J Med 87:468–471
20. Boelaert JR, Fenves AZ, Coburn JW (1991) Deferoxamine therapy and mucormycosis in dialysis patients: report of an international registry. Am J Kidney Dis 18:660–667
21. Sugar AM (1992) Mucormycosis. Clin Infect Dis 14(Suppl 1):S126–S129
22. McNab AA, McKelvie P (1997) Iron overload is a risk factor for zygomycosis. Arch Ophthalmol 115:919–921
23. Marty FM, Cosimi LA, Baden LR (2004) Breakthrough mucormycosis after voriconazole treatment in recipients of hematopoietic stem-cell transplants. N Engl J Med 350:950–952
24. Oren I (2005) Breakthrough mucormycosis during empirical voriconazole therapy in febrile patients with neutropenia. Clin Infect Dis 40:770–771
25. Bouza E, Muñoz P, Guinea J (2006) Mucormycosis: an emerging disease? Clin Microbiol Infect 12(suppl 7):7–23
26. Petrikkos GL, Skiada A et al (2003) Mucormycosis: ten year experience in a tertiary-care centre in Greece. Eur J Clin Microbiol Infect Dis 22:753–756
27. Antoniadou A (2009) Outbreaks of mucormycosis in hospitals. Clin Microbiol Infect 15(Suppl 5):55–59
28. Cheng VC, Chan JF et al (2009) Outbreak of intestinal infection due to *Rhizopus microsporus*. J Clin Microbiol 47:2834–2843
29. Spellberg B, Edwards J Jr, Ibrahim A (2005) Novel perspectives on mucormycosis: pathophysiology, presentation, and management. Clin Microbiol Rev 18:556–569
30. Alom S, Ali F, Md ZK (2021) A comprehensive review on mucormycosis (black fungus) and its association with covid-19. Curr Trends Pharm Res 8(1):11–40
31. Zaoutis TE, Roilides E et al (2007) Zygomycosis in children: a systematic review and analysis of reported cases. Pediatr Infect Dis J 26:723–727
32. Gorbalenya AE, Baker SC et al (2020) The species severe acute respiratory syndrome related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. Nat Microbiol 5:536–544
33. Zhou P, Yang XL et al (2020) A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature 579(7798):270–273
34. Wang Y, Wang Y et al (2020) Unique epidemiological and clinical features of the emerging 2019 novel coronavirus pneumonia (COVID 19) implicate special control measures. J Med Virol 92(6):568–576
35. Yang X, Yu Y et al (2020) Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. Lancet Respir Med 8(5):475–481
36. Mahalaxmi I, Jayaramayya K et al. (2021) Mucormycosis: an opportunistic pathogen during COVID-19 environmental research 201 (2021) 111643. https://doi.org/10.1016/j.envres.2021.111643
37. Gangneux JP, Bougnoux ME et al (2020) Invasive fungal diseases during COVID-19: we should be prepared. Journal de mycologie medicale 30(2):100971
38. Agarwal A, Puri I (2021) Mucormycosis in post covid patients: a systematic review. FASEB J 35. https://doi.org/10.1096/fasebj.2021.35.S1.04770
39. Garg D, Muthu V et al (2021) Coronavirus disease (Covid-19) associated mucormycosis (CAM): case report and systematic review of literature. Mycopathologia 186(2):289–298
40. Sharma S, Grover M et al (2021) Post coronavirus disease mucormycosis: a deadly addition to the pandemic spectrum. J Laryngol Otol 135(5):442–447

41. Khatri A, Chang KM et al (2021) Mucormycosis after coronavirus disease 2019 infection in a heart transplant recipient - case report and review of literature. J Mycol Med 31(2):101125. https://doi.org/10.1016/j.mycmed.2021.101125
42. Soman R, Sunavala A (2021) Post COVID-19 Mucormycosis—from the frying pan into the fire. J Assoc Physicians India 69(1):13–14
43. Honavar SG (2021) Code mucor: guidelines for the diagnosis, staging and management of Rhino-Orbito-Cerebral mucormycosis in the setting of COVID-19. Indian J Ophthalmol 69: 1361–1365
44. Diamond RD, Haudenschild CC, Erickson NF 3rd. (1982) Monocyte-mediated damage to Rhizopus oryzae hyphae in vitro. Infect Immun 38:292–297
45. Waldorf AR (1989) Pulmonary defense mechanisms against opportunistic fungal pathogens. Immunol Ser 47:243–271
46. Waldorf AR, Ruderman N, Diamond RD (1984) Specific susceptibility to mucormycosis in murine diabetes and bronchoalveolar macrophage defense against Rhizopus. J Clin Invest 74: 150–160
47. Ibrahim AS, Spellberg B et al (2005) *Rhizopus oryzae* adheres to, is phagocytosed by, and damages endothelial cells in vitro. Infect Immun 73:778–783
48. Boelaert JR, de Locht M et al (1993) Mucormycosis during deferoxamine therapy is a siderophore mediated infection. In vitro and in vivo animal studies. J Clin Invest 91:1979–1986
49. Netea MG, Van der Meer JW, Kullberg BJ (2006) Role of the dual interaction of fungal pathogens with pattern recognition receptors in the activation and modulation of host defence. Clin Microbiol Infect 12:404–409
50. Ibrahim AS, Spellberg B et al (2012) Pathogenesis of mucormycosis. Clin Infect Dis 54(suppl 1):S16–S22
51. Chamilos G, Lewis RE et al (2008) Zygomycetes hyphae trigger an early, robust proinflammatory response in human polymorphonuclear neutrophils through toll-like receptor 2 induction but display relative resistance to oxidative damage. Antimicrob Agents Chemother 52:722–724
52. Liles WC, Huang JE et al (1997) Granulocyte colony-stimulating factor administered in vivo augments neutrophil- mediated activity against opportunistic fungal pathogens. J Infect Dis 175:1012–1015
53. Petraitis V, Petraitiene R et al (2013) Increased virulence of Cunninghamella bertholletiae in experimental pulmonary mucormycosis: correlation with circulating molecular biomarkers, sporangiospore germination and hyphal metabolism. Med Mycol 51:72–82
54. Simitsopoulou M, Georgiadou E et al (2010) *Cunninghamella bertholletiae* exhibits increased resistance to human neutrophils with or without antifungal agents as compared to Rhizopus spp. Med Mycol 48:720–724
55. Ibrahim A, Spellberg B, Edwards Jr J (2008) Iron acquisition: a novel prospective on mucormycosis pathogenesis and treatment. Curr Opin Infect Dis 21(6):620–625
56. De Vlieger G, Lagrou K, Maertens J (2011) Beta-D-glucan detection as a diagnostic test for invasive aspergillosis in immunocompromised critically ill patients with symptoms of respiratory infection: an autopsy-based study. J Clin Microbiol 49(11):3783–3787
57. Spellberg B, Kontoyiannis DP, Fredricks D (2012) Risk factors for mortality in patients with mucormycosis. Med Mycol 50:611–618
58. Baldin C, Ibrahim AS (2017) Molecular mechanisms of mucormycosis-the bitter and the sweet. PLoS Pathog 13(8):e1006408
59. Garre V (2022) Recent advances and future directions in the understanding of mucormycosis. Front Cell Infect Microbiol 12:850581
60. Samson R, Dharne M (2022) COVID-19 associated mucormycosis: evolving technologies for early and rapid diagnosis. 3Biotech 12:6. https://doi.org/10.1007/s13205-021-03080-4
61. Boelaert JR, van Roost GF et al (1988) The role of desferrioxamine in dialysis associated mucormycosis: report of three cases and review of the literature. Clin Nephrol 29:261–266

62. Artis WM, Fountain JA et al (1982) A mechanism of susceptibility to mucormycosis in diabetic ketoacidosis: transferrin and iron availability. Diabetes 31:1109–1114
63. Shoham S, Levitz SM (2005) The immune response to fungal infections. Br J Haematol 129: 569–582
64. Waldorf AR, Levitz SM, Diamond RD (1984) In vivo bronchoalveolar macrophage defense against Rhizopus oryzae and aspergillus fumigatus. J Infect Dis 150:752–760
65. Kontoyiannis DP, Lewis RE (2011) How I treat mucormycosis. Blood 118:1216–1224
66. Chinn RY, Diamond RD (1982) Generation of chemotactic factors by *Rhizopus oryzae* in the presence and absence of serum: relationship to hyphal damage mediated by human neutrophils and effects of hyperglycemia and ketoacidosis. Infect Immun 38:1123–1129
67. Lamaris GA, Ben-Ami R et al (2009) Increased virulence of zygomycetes organisms following exposure to voriconazole: a study involving fly and murine models of zygomycosis. J Infect Dis 199:1399–1406
68. Kontoyiannis DP, Lionakis MS et al (2005) Zygomycoses in a tertiary-care cancer center in the era of Aspergillus-active antifungal therapy: a case-control observational study of 27 recent cases. J Infect Dis 191:1350–1360
69. Soliman SSM, Baldin C et al (2021) Mucoricin is a Ricin-like toxin that is critical for the pathogenesis of mucormycosis. Nat Microbiol 6:313–326
70. Chakrabarti A, Kumar P et al (1997) Primary cutaneous zygomycosis due to *Saksenaea vasiformis* and *Apophysomyces elegans*. Clin Infect Dis 24:580–583
71. Espinel-Ingroff A, Oakley LA, Kerkering TM (1987) Opportunistic zygomycotic infections. Mycopathologia 97:33–41
72. Sugar AM (2000) Agents of mucormycosis and related species. In: Mandell GI, Bennett JE, Dolin R (eds) Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, 5th edn. Churchill Livingstone, New York, pp 2685–2695
73. Chakrabarti A, Das A et al (2001) Ten years' experience in zygomycosis at a tertiary care centre in India. J Infect 42:261–266
74. Chadli-Chaieb M, Bchir A et al (2005) Mucormycosis in the diabetic patient. Presse Med 34: 218–222
75. McNulty JS (1982) Rhinocerebral mucormycosis: predisposing factors. Laryngoscope 92: 1140–1143
76. Ryan M, Yeo S et al (2001) Rhinocerebral zygomycosis in childhood acute lymphoblastic leukaemia. Eur J Pediatr 160:235–238
77. Wehl G, Hoegler W et al (2002) Rhinocerebral mucormycosis in a boy with recurrent acute lymphoblastic leukemia: long-term survival with systemic antifungal treatment. J Pediatr Hematol Oncol 24:492–494
78. Effat KG, Karam M, El-Kabani A (2005) Pott's puffy tumour caused by mucormycosis. J Laryngol Otol 119:643–645
79. Parfrey NA (1986) Improved diagnosis and prognosis of mucormycosis. A clinicopathologic study of 33 cases. Medicine (Baltimore) 65:113–123
80. Ferry AP, Abedi S (1983) Diagnosis and management of rhinoorbitocerebral mucormycosis (phycomycosis). A report of 16 personally observed cases. Ophthalmology 90:1096–1104
81. Yohai RA, Bullock JD et al (1994) Survival factors in rhino-orbital-cerebral mucormycosis. Surv Ophthalmol 39:3–22
82. Dhiwakar M, Thakar A, Bahadur S (2003) Improving outcomes in rhinocerebral mucormycosis—early diagnostic pointers and prognostic factors. J Laryngol Otol 117:861–865
83. Fermanis GG, Matar KS, Steele R (1991) Endobronchial zygomycosis. Aust N Z J Surg 61: 391–393
84. Husari AW, Jensen WA et al (1994) Pulmonary mucormycosis presenting as an endobronchial lesion. Chest 106:1889–1891
85. Vaideeswar P (2002) Fatal hemoptysis due to mucormycotic intrapulmonary arterial aneurysm. Int J Cardiol 83:273–274

86. Dubey A, Patwardhan RV et al (2005) Intracranial fungal granuloma: analysis of 40 patients and review of the literature. Surg Neurol 63:254–260
87. Sehgal A, Raghavendran M et al (2004) Rhinocerebral mucormycosis causing basilar artery aneurysm with concomitant fungal colonic perforation in renal allograft recipient: a case report. Transplantation 78:949–950
88. Cuadrado LM (1996) Cerebral mucormycosis associated with intravenous drug use. Clin Infect Dis 22:198
89. Leen CL, Brettle RP (1991) Fungal infections in drug users. J Antimicrob Chemother 28(suppl A):83–96
90. Vera A, Hubscher SG et al (2002) Invasive gastrointestinal zygomycosis in a liver transplant recipient: case report. Transplantation 73:145–147
91. Calle S, Klatsky S (1966) Intestinal phycomycosis (mucormycosis). Am J Clin Pathol 45:264–272
92. Richardson MD, Warnock DW (2003) Mucormycosis. In: Richardson MD, Warnock DW (eds) Fungal infection diagnosis and management. Blackwell, Oxford, United Kingdom, pp 230–240
93. Nosari A, Oreste P et al (2000) Mucormycosis in hematologic malignancies: an emerging fungal infection. Haematologica 85:1068–1071
94. Pagano L, Offidani M et al (2004) Mucormycosis in hematologic patients. Haematologica 89: 207–214
95. Ingram CW, Sennesh J et al (1989) Disseminated zygomycosis: report of four cases and review. Rev Infect Dis 11:741–754
96. Walsh TJ, Gamaletsou MN et al (2012) Early clinical and laboratory diagnosis of invasive pulmonary, extrapulmonary, and disseminated mucormycosis (zygomycosis). Clin Infect Dis 54(suppl 1):S55–S60
97. Skiada A, Lass-Floerl C et al (2018) Challenges in the diagnosis and treatment of mucormycosis. Med Mycol 56:S93–S101
98. Frater JL, Hall GS, Procop GW (2001) Histologic features of zygomycosis: emphasis on perineural invasion and fungal morphology. Arch Pathol Lab Med 125:375–378
99. Tille PM. (2014) Hyaline molds, mucorales (zygomycetes), dermatophytes, and opportunistic and systemic mycoses. In Bailey and Scott's diagnostic microbiology, 13th ed. New York: Mosby. Pp. 732–758
100. Ramani R, Gromadzki S et al (1998) Efficacy of API 20C and ID 32C systems for identification of common and rare clinical yeast isolates. J Clin Microbiol 36:3396–3398
101. Schwarz P, Lortholary O et al (2007) Carbon assimilation profiles as a tool for identification of zygomycetes. J Clin Microbiol 45:1433–1439
102. Tarrand JJ, Lichterfeld M et al (2003) Diagnosis of invasive septate mold infections. A correlation of microbiological culture and histologic or cytologic examination. Am J Clin Pathol 119:854–858
103. McLean FM, Ginsberg LE, Stanton CA (1996) Perineural spread of rhinocerebral mucormycosis. Am J Neuroradiol 17:114–116
104. Guarner J, Brandt ME (2011) Histopathologic diagnosis of fungal infections in the 21st century. Clin Microbiol Rev 24:247–280
105. Lamoth F, Alexander BD. (2014) Nonmolecular methods for the diagnosis of respiratory fungal infections. In Clinics in laboratory medicine. Vol 34. W.B. Saunders. United States of America. pp. 315–36
106. Cornely OA, Alastruey-Izquierdo A et al (2019) Global guideline for the diagnosis and management of mucormycosis: an initiative of the European Confederation of Medical Mycology in cooperation with the Mycoses Study Group Education and Research Consortium. Lancet Infect Dis 19:e405–e421
107. Jensen HE, Salonen J, Ekfors TO (1997) The use of immunohistochemistry to improve sensitivity and specificity in the diagnosis of systemic mycoses in patients with hematological malignancies. J Pathol 181:100–105

108. Son HJ, Song JS et al (2020) A comparison of histomorphologic diagnosis with culture- and immunohistochemistry-based diagnosis of invasive aspergillosis and mucormycosis. Infect Dis 52:279–283
109. Balajee SA, Sigler L, Brandt ME (2007) DNA and the classical way: identification of medically important molds in the 21st century. Med Mycol 45:475–490
110. Ling H, Yuan Z et al (2014) Accuracy of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of clinical pathogenic fungi: a meta-analysis. J Clin Microbiol 52(7):2573–2582
111. Blauwkamp TA, Thair S et al (2019) Analytical and clinical validation of a microbial cell-free DNA sequencing test for infectious disease. Nat Microbiol 4(4):663–674
112. Zaman K, Rudramurthy SM et al (2017) Molecular diagnosis of rhino-orbito-cerebral mucormycosis from fresh tissue samples. J Med Microbiol 66:1124–1129
113. Alanio A, Garcia-Hermoso D et al (2015) Molecular identification of mucor in human tissues: contribution of PCR electrospray-ionization mass spectrometry. Clin Microbiol Infect 21:594.e1–594.e5
114. Lengerova M, Racil Z et al (2014) Rapid detection and identification of Mucormycetes and bronchoalveolar lavage samples from immunocompromised patients with pulmonary infiltrates by use of high-resolution melt analysis. J Clin Microbiol 52:2824–2828
115. Dadwal SS, Kontoyiannis DP (2018) Recent advances in the molecular diagnosis of mucormycosis. Expert Rev Mol Diagn 18:845–854
116. Dannaoui E (2009) Molecular tools for identification of zygomycetes and the diagnosis of zygomycosis. Clin Microbiol Infect 15(suppl 5):66–70
117. Guegan H, Iriart X et al (2020) Evaluation of MucorGenius® mucorales PCR assay for the diagnosis of pulmonary mucormycosis. J Infect 81:311–317
118. Mercier T, Reynders MB et al (2019) Serial detection of circulating mucorales DNA in invasive mucormycosis: a retrospective multicenter evaluation. J Fungi 5:113
119. Georgiadou SP, Sipsas NV et al (2011) The diagnostic value of halo and reversed halo signs for invasive mold infections in compromised hosts. Clin Infect Dis 52:1144–1155
120. Liu Y, Wu H et al (2013) Utility of 18F- FDG PET/CT in diagnosis and management of mucormycosis. Clin Nucl Med 38:e370–e371
121. Nair V, Sharma RK et al (2017) Pulmonary mucormycosis diagnosed by convex probe endobronchial ultrasound-guided fine needle aspiration of cavity wall. Lung India 34:179–181
122. Potenza L, Vallerini D et al (2011) Mucorales-specific T cells emerge in the course of invasive mucormycosis and may be used as a surrogate diagnostic marker in high-risk patients. Blood 118:5416–5419
123. Koshy S, Ismail N et al (2017) Breath-based diagnosis of invasive mucormycosis (IM). Open Forum Infect Dis 4:S53–S54
124. Kasai M, Harrington SM et al (2008) Detection of molecular biomarkers for *Rhizopus spp., Mucor spp*., and *Cunninghamella spp*. by quantitative PCR and melt curve analysis in plasma, bronchoalveolar lavage, and lung tissue in experimental pulmonary zygomycosis. J Clin Microbiol 46:3690–3702
125. Chamilos G, Lewis RE, Kontoyiannis DP (2008) Delaying amphotericin B-based frontline therapy significantly increases mortality among patients with hematologic malignancy who have zygomycosis. Clin Infect Dis 47(4):503–509
126. Riley TT, Muzny CA et al (2016) Breaking the Mold. Ann Pharmacother 50(9):747–757
127. Ma LJ, Ibrahim AS et al (2009) Genomic analysis of the basal lineage fungus *Rhizopus oryzae* reveals a whole-genome duplication. PLoS Genet 5:e1000549
128. Spellberg B, Walsh TJ et al (2009) Recent advances in the management of mucormycosis: from bench to bedside. Clin Infect Dis 48:1743–1751
129. Spellberg B, Ibrahim AS (2010) Recent advances in the treatment of mucormycosis. Curr Infect Dis Rep 12:423–429
130. Dannaoui E, Meletiadis J et al (2003) In vitro susceptibilities of zygomycetes to conventional and new antifungals. J Antimicrob Chemother 51:45–52

131. Sun QN, Fothergill AW et al (2002) In vitro activities of posaconazole, itraconazole, voriconazole, amphotericin B, and fluconazole against 37 clinical isolates of zygomycetes. Antimicrob Agents Chemother 46:1581–1582
132. Lewis RE, Lortholary O et al (2012) How does antifungal pharmacology differ for mucormycosis versus aspergillosis? Clin Infect Dis 54(suppl 1):S67–S72
133. Spellberg B, Fu Y et al (2005) Combination therapy with amphotericin B lipid complex and caspofungin acetate of disseminated zygomycosis in diabetic ketoacidotic mice. Antimicrob Agents Chemother 49:830–832
134. Chassaing N, Valton L et al (2003) Rhino-cerebral fungal infection successfully treated with supplementary hyperbaric oxygen therapy. Rev Neurol (Paris) 159:1178–1180
135. John BV, Chamilos G, Kontoyiannis DP (2005) Hyperbaric oxygen as an adjunctive treatment for zygomycosis. Clin Microbiol Infect 11:515–517
136. Abzug MJ, Walsh TJ (2004) Interferon-gamma and colony-stimulating factors as adjuvant therapy for refractory fungal infections in children. Pediatr Infect Dis J 23:769–773
137. Gil-Lamaignere C, Simitsopoulou M et al (2005) Interferon gamma and granulocyte-macrophage colony stimulating factor augment the activity of polymorphonuclear leukocytes against medically important zygomycetes. J Infect Dis 191:1180–1187
138. Grimaldi D, Pradier O et al (2017) Nivolumab plus interferon-gamma in the treatment of intractable mucormycosis. Lancet Infect Dis 17(1):18
139. Gebremariam T, Wiederhold NP et al (2015) VT-1161 protects immunosuppressed mice from *Rhizopus arrhizus var. arrhizus* infection. Antimicrob Agents Chemother 59:7815–7817
140. Salem SS, Ali OM et al (2022) Pseudomonas indica-mediated silver nanoparticles: antifungal and antioxidant biogenic tool for suppressing mucormycosis fungi. J Fungi 8:126
141. Elfiky Abdo A, Ibrahim MN (2022) The antiviral polymerase inhibitor against Mucormycosis *in silico* perspective. Res J Biotech 17(4):114–115
142. Banik A, Sheikh RA et al (2022) Targeting mucormycosis polyprotein (RVT_1 region) through antifungal phytochemicals: an in-silico perspective. Inform Med Unlocked 29: 100851. https://doi.org/10.1016/j.imu.2022.100851
143. Hashem AH, Shehabeldine AM et al (2022) Antifungal activity of endophytic aspergillus terreus extract against some fungi causing mucormycosis: ultrastructural study. Appl Biochem Biotechnol 2:1–15
144. Kontoyiannis DP, Lewis RE et al (2012) Future directions in mucormycosis research. Clin Infect Dis 54(S1):S79–S85

5 Microplastic Pollution: Sources, Environmental Hazards, and Mycoremediation as a Sustainable Solution

Ashish Bhatt, Darshankumar Prajapati, Akshaya Gupte, and Shilpa Gupte

Abstract

Synthetic polymers have replaced natural polymers due to their low production cost, wide range of applications, and better resistant properties. Plastic polymers are the most preferred one among synthetic polymers as they have high tolerance against high temperature, photooxidation, and chemical degradation. While these properties make them desirable compound for manufacturing various products, it also imposes environmental hazards upon releasing microplastic particles which has negative impact on terrestrial as well as aquatic ecosystems. Although many plastic remediation technologies involving physicochemical methods have emerged in past decades, they have been inefficient when it comes to remediating microplastic pollutants. In the present scenario, mycoremediation approach has been suggested as an alternative green approach to remediate the microplastic pollution. Fungi possess diverse enzyme systems, metabolic processes, and bioactive compounds which are proven to be efficient in removing microplastic pollutants. The present review highlights the detailed mechanisms as well as evidences for microplastic remediation by fungal system. The types of microplastic pollutants, source, and their hazardous effects on various ecosystems have also been compiled.

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Keywords

Microplastics · Polyethylene · Polypropylene · Mycoremediation · Diketone · Terephthalate · Antioxidants

5.1 Introduction

Plastic materials are one of the most important synthetic compounds produced by the chemical industries that have countless applications in the daily life of human beings [1]. The history of using plastic material goes back to as far as 1600 BC with the use of balls, figurines, and bands from natural rubber by Mesoamerican civilization [2]. The unique properties of plastic materials such as temperature tolerance, chemical and light resistance, and molding capability despite the toughness along with low production costs have constantly driven their demands with maximum being for the low-density polyethylene (LDPE) and high-density polyethylene (HDPE) plastics followed by polypropylene (PP) plastics, polyvinyl chloride (PVC) plastics, polystyrene (PS), and polyethylene terephthalate (PET) plastics [3]. In general, plastic materials are recycled or disposed in landfill and waste treatment plants. However, a substantial amount of plastic waste still contaminates the environment because of inefficient treatment, improper way of disposal, or loss with waste effluents [4].

Microplastics are one of the most persistent chemicals known to contaminate various environmental niches. These can be found in the water, soil, and air in a direct particulate form or indirectly in the ecosystem due to their small (5 mm) particle size [5, 6]. Primarily, microplastics are produced by industries for manufacturing various plastic products, and among the types of plastics, PE and PP plastics make up the major portion of it [7]. In fact, the current annual plastic production is nearly 400 million tons globally and is expected to rise beyond 800 million tons in the next 30 years. This rise is a major concern from the environmental safety perspective. Low capital costs and labor requirements have further pushed up the industries to use more plastics in manufacturing household and luxury products including toys, kitchen appliances, furniture, electronic products, and sanitary goods.

The excessive use of plastics can be the reason as to why microplastic pollutants have also been detected even at the places of earth with low human activity. Microplastic pollution can lead to widespread detrimental physicochemical effects and their long-term biological impacts are still under study. Furthermore, various distant and untouched ecosystems and diverse range of species residing in them can be potentially harmed due to the collective effects of anthropogenic activity and climate-induced stress [8].

The current physical and chemical remediation methods of microplastic removal (recycling, chemical degradation, and incineration) are not sustainable solutions to microplastic waste. These methods become more inefficient when microplastic is littered and spreads out in different ecosystems. In the past two decades, fungal remediation has been suggested as long-term and ecofriendly solution of

microplastic pollution [9]. Fungi are known to have natural abilities to invade plastic materials by using various groups of enzymes that have the capability to remediate pollutants and act on nonspecific substrates. Moreover, fungi can produce unique surface coating natural compounds called hydrophobins that can help hyphae to attach and penetrate three-dimensional substrates [10]. Hence, for the long-term prospective, mycoremediation has more potential to be an effective microplastic remediation strategy.

5.2 Fungi-Mediated Remediation

Microorganisms produce a wide array of enzymes as part of their metabolic activities, and these enzymes have potential to affect physicochemical properties in plastic polymer structure which may eventually assist in its degradation. The specific enzymes involved in plastic degradation require detailed investigations further.

Most commonly reported enzymes (laccases, esterases, and peroxidases) for plastic degradation have either oxidative or hydrolytic activities. For example, in a consortium study involving the use of *Ascomycetes* for the degradation LDPE, ligninolytic enzyme activities were observed [11]. Similar results were also reported by other researcher [12, 13], where other *Ascomycete* fungal species (*Zalerion maritimum* and *Gloeophyllum trabeum*) were capable of degrading microplastic materials. Many studies have indicated that laccase has a significant depolymerizing activity against HDPE by the oxidative cleavage mechanism where amorphous region is targeted carbonyl functional groups accessible for further degradation within the polymer chain [14, 15]. According to Restrepo-Flórez et al. [16], fungal enzymes are capable of reducing the length of PE polymer change, and once the reaction starts, it can further degrade by more metabolic activities. El-Morsy et al. [17] reported activities of protease, lipase, and esterase enzyme in polyurethane plastic material by *Monascus* sp. strains. As per Danso et al. [18] and Liebminger et al. [19], serine hydrolases (cutinases, carboxylesterases, and lipases) and polyesterases are involved in the PET degradation. Among the polyester degrading microbial enzymes, polyester hydrolases, cutinases, and their homologues were shown to have potential for PET degradation [15]. Recent studies have focused more on discovering alkane hydrolase due to its specific degradation activity against PE [20, 21]. The information regarding enzymes that play a role in hydrolysis of PP and PU is still unknown, and therefore, it requires further investigations [22]. Figure 5.1 presents a general scheme of enzymatic degradation of polymers.

5.2.1 Types of Microplastics

According to Law and Thompson [23], microplastics can be simply defined as water-insoluble, solid synthetic plastic particles with size ranging from 1 μm to 5 mm. The insolubility in water decreases the bioavailability of microplastics which

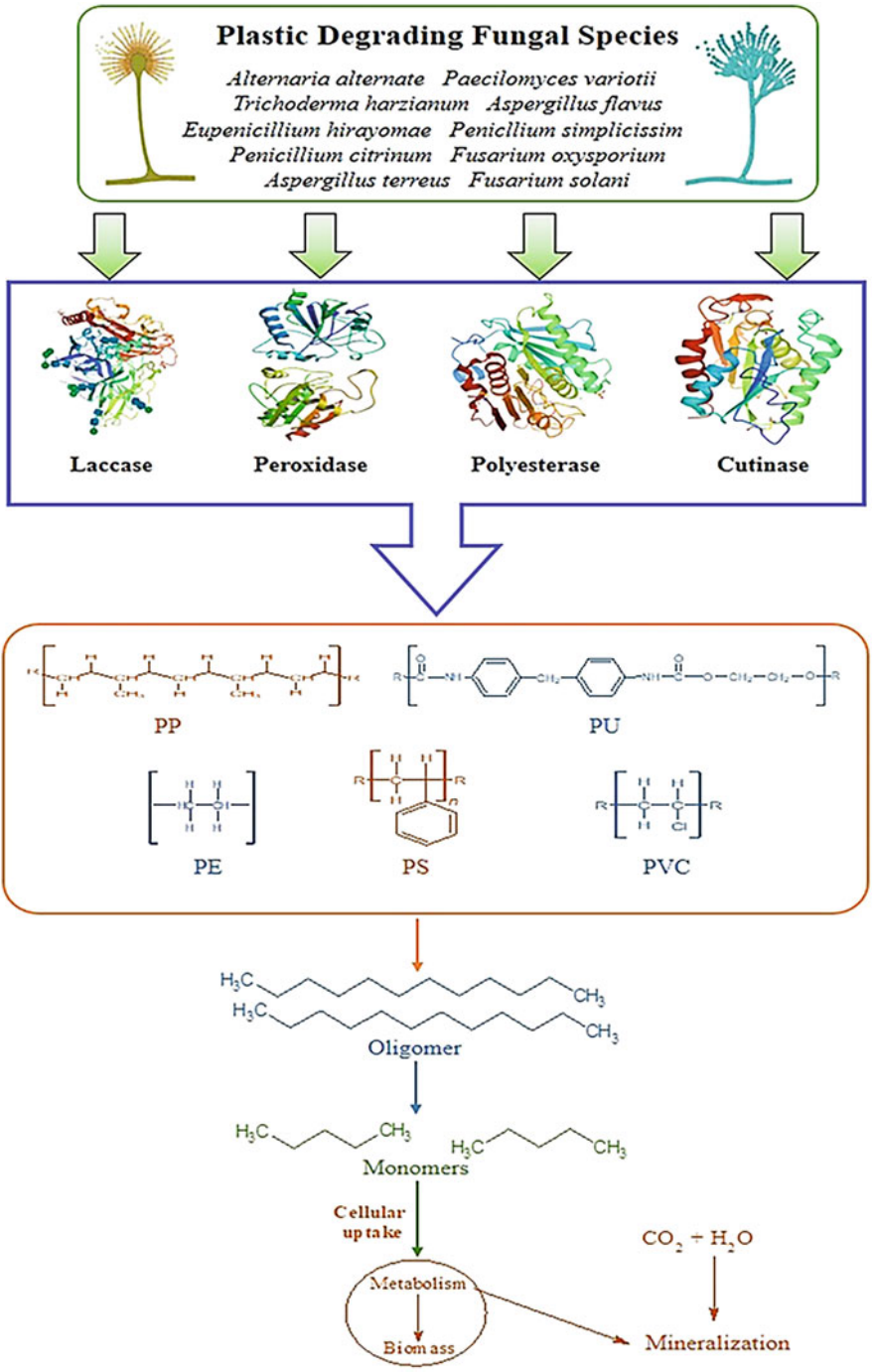

Fig. 5.1 Enzymatic actions on polymers and their fate

in turn hampers their biological degradation. Furthermore, longer persistence of microplastics increases the chances of their biomagnification and bioaccumulation. Therefore, microplastics can impose drastic effects on the exposed organisms by physical or chemical means. Physical effects are associated with their concentration, shape, and particle size, while chemical effects are related to hazardous chemicals used in the synthesis of plastics polymers.

The following are the commonly used types of microplastics with known biological and environmental hazards:

5.2.1.1 Polyethylene (PE)

Polyethylene is a linear hydrocarbon polymer with ethylene (C_2H_4) as monomer, which is mostly used in manufacturing bottles, containers, and plastics bags. It is synthesized by cleavage of double bond followed by coupling of ethylene monomers in either straight or branched manner. Resulted polymer (Fig. 5.2) is highly hydrophobic in nature and cannot pass through microbial cell membranes because of its large size [24].

Considering the number of carbon atoms in the formulation stage, PE can be divided into very-low-density polyethylene, low-density polyethylene (LDPE), linear low-density polyethylene, high-density polyethylene (HDPE), high molecular weight HDPE, and ultrahigh molecular weight HDPE [25, 26]. HDPE are resistant to solvents including allyl alcohol, benzene, aniline, ethyl acetate, isopropyl alcohol, and methanol due to their superior density to strength ratio which indicates stronger intramolecular force. LDPE can tolerate strong acids (acetic acid and hydrochloric acid), alcohols (methanol and ethanol), as well as alkali (ammonia and urea) due to its low density and branched structure [27, 28]. Furthermore, tackling pollution from polyethylene terephthalate (PET), a modified form of polyethylene, is even more challenging due to its durability, strength, flexibility, hydrophobicity, and high crystallinity and thermoplasticity [29].

5.2.1.2 Polystyrene (PS)

Polystyrene (Fig. 5.3) is an aromatic homopolymer synthesized by polymerization of styrene monomers. Styrene monomers are synthesized by condensation of ethylene and benzene followed by catalytic dehydrogenation [30]. PS is a durable thermoplastic which is used in production of CDs, toys, electric wire coatings, toothbrushes, etc. One of the most abundantly generated products from PS is Styrofoam, which is

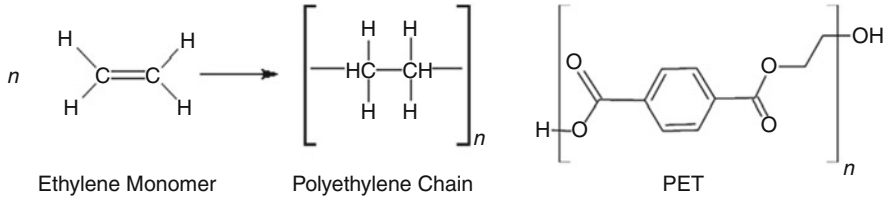

Fig. 5.2 Structure of PE and PET

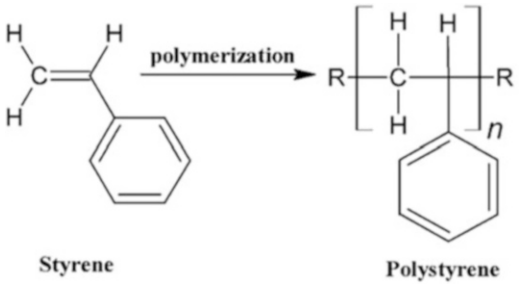

Fig. 5.3 Structure of PS

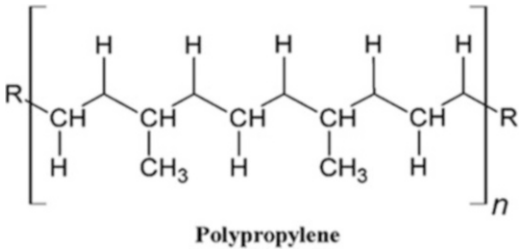

Fig. 5.4 Structure of PP

widely used as a packaging material as well as shock absorbers for food storage containers and transport of goods [31].

PS is one of the most thermostable plastics (no degradation up to 200 °C temperature) occurring in three forms: expanded, crystalline, and high-impact [32]. PS becomes a major concern because of its leaching problem in water bodies in the form of micro- and nanoplastics. Apart from this, its monomers, plasticizers, and additives are also known to be toxic toward living beings [33]. According to the Environmental Protection Agency (EPA), 300 ppm (1000 μg/m^3) styrene is considered as a chronic case of exposure. As per the guidelines of WHO, 2016 [34], styrene levels in polymer industry waste should not exceed 20 ppm, but even at this concentration, its prolonged exposure and biomagnification may lead to disastrous consequences for the environment.

5.2.1.3 Polypropylene (PP)

Polypropylene (Fig. 5.4) is a saturated polymer linear hydrocarbon chain, synthesized by polymerization of propylene (propene) monomers [35, 36]. The synthesis process is reminiscent to polystyrene which is by catalytic dehydrogenation of propylene. In comparison to PE and PS, PP is lighter, stronger, and more transparent in its native form. There are three forms of PP: amorphous, semicrystalline, and crystalline, with commercial one being mixture of 75% semicrystalline and 25% amorphous [37].

Due to its versatility, PP is used in manufacturing of injection and expansion molding as well as packaging of various food products. PP is completely resistant to water vapors, oils, and fats which decreases its bioavailability and prospects of natural degradation. The recent emergence of COVID-19 pandemic has dramatically

increased the demand for personal protective equipment (PPE) kits, which are mainly composed of PP plastic [38, 39].

5.2.1.4 Polyvinyl Chloride (PVC)

Polyvinyl chloride is synthesized by polymerization of vinyl chloride monomer (Fig. 5.5). The monomer vinyl chloride is a result of chlorination of acetylene by hydrochloric acid. PVC is the third most produced plastic material globally with nearly 3.2% increase in global demand [40]. PVC is available in rigid as well as flexible forms [37]. Rigid PVC is commonly used in doors, pipes, windows, and packing, whereas flexible form is used in plumbing and imitation leather and as an alternate to rubbers and canvas [41, 42].

PVC plastics are chemically inert and show resistance to acids and inorganic chemicals. Furthermore, chloride molecules in monomer give it protection against oxidative damage and fire [37]. Because of these physicochemical properties, PVC plastics persist for longer period of time and may magnify eventually. Additionally, plasticizers (up to 70%) and additives are required to be added during PVC manufacturing to deal with its brittleness which makes PVC materials more hazardous to the environment [43].

5.2.1.5 Polyurethane (PU)

In terms of monomeric units used in polymerization, polyurethane is different in comparison with PE, PP, and PS. PU is a heteropolymer consisting of copolymeric segment of repeating units (Fig. 5.6). For the synthesis of PU, diisocyanate, diols, and chain extenders are used as precursors. The unique property of polyurethane is that one of the segments is crystalline in nature that affords hardness, whereas the other segment is malleable which contributes to its flexibility and softness. The resultant flexibility and softness of the final polymer is due to the presence of diols [44]. Being biologically and chemically inert and mechanically strong, PU plastics

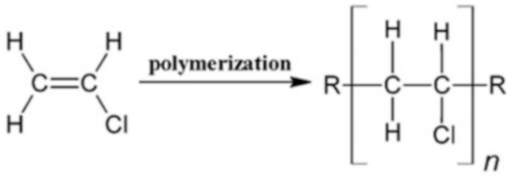

Fig. 5.5 Structure of PVC

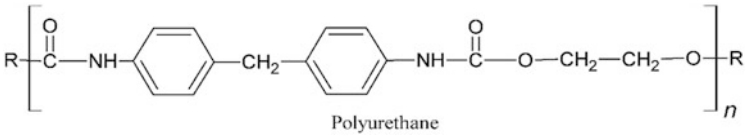

Fig. 5.6 Structure of PU

are used in medical devices (pacemakers, injectable drips, etc.), construction materials, and coatings as well as in automotive manufacturing [45].

The major issue with PU materials is the higher combustibility of raw material itself and additives that are used for plastic manufacturing. Upon combustion, PU releases highly concentrated fumes of HCN, HCNO, CO, NO, NO_2, and other toxic gases, which are highly harmful to the living organisms [46].

5.2.2 Additives in Plastics

For the manufacturing of microplastics, two types of chemicals are used: (a) monomeric/oligomeric raw materials and additive compounds and (b) chemicals that may be absorbed from the surrounding environment [47]. Additives used in the production of plastics such as plasticizers, UV stabilizers, dyes, antioxidants, moisture repellents, lubricants, and flame retardants are of major concern due as they are able to impart toxicity to diverse range of environmental niches at low concentrations [48]. Additives are the chemicals that are supplemented along with raw material to provide transparency and color. Additionally, they also assist in enhancing the resistance to radiation, light, ozone, water, and microbial degradation as well as in improving thermal, electrical, and mechanical resistance [49].

5.2.2.1 Plasticizers

Plasticizers are one of the major health concerns due to their interaction with the biological systems especially interfering with normal cellular functionality [50]. Plasticizers are the organic compounds that are used for shear reduction in the mixing stage of polymer production and also employed to increase resistance in the resultant plastic film. Moreover, plasticizers also contribute to flexibility, durability, and tensile strength to polymeric films [51]. Some of the commonly used plasticizers are DPP, DOA, DEHA, diisobutyl phthalate, DEP, phthalic esters, ATBC, and DBP. They are mainly used in production of PVC and PET plastics [52].

5.2.2.2 Flame Retardants and Thermal Stabilizers

Plastic polymers are generally inflammable in nature due to formation of volatile compounds because of breaks in polymer chain. Flame retardants are one of the important classes of chemical compounds that help with overcoming this problem [53]. Additionally, flame retardants also contribute to increasing thermal stability. The commonly employed flame retardants are aluminum hydroxide, tetrabromobisphenol A, and hexabromocyclododecane [54, 55].

The thermal stability and heat tolerance of plastic materials are enhanced by adding thermal stabilizers. Primary heat stabilizers include metal salt blends, lead compounds, and organotin compounds, and secondary heat stabilizers include epoxy compounds alkyl organophosphites, and diketones are used as secondary heat stabilizers [51]. Apart from this, blending of polymers such as vinyl copolymer, PVC, LDPE, PVDC, and polyamides is also used as thermal stabilizers [48].

5.2.2.3 Antioxidants

Plastic products have mass appeals due to their resistance toward oxidative agents and reactive oxygen species (ROS), and this property is further enhanced by adding antioxidants. Antioxidants are used as additives in many synthetic polymers including polyethylene and polypropylene, which are more than 60% of global demand for antioxidant additives [56]. Antioxidants also play a role in preventing aging and degradation of plastics by delaying oxidation. Commonly used antioxidants are octadecyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate, pentaerythrityl-tetrakis-3-(3,5-di-tert-butyl-4-hydroxyphenyl) propionate, and 2,4-di-tert-butylphenol [57]. Although antioxidant compounds improve chemo-stability of plastics, they may leach out into water bodies upon exposure [58].

5.2.3 Environmental Hazards Due to Microplastics

Anthropogenic activities have caused irreversible environmental changes which have caused disruption of normal functioning of various ecosystems and also threatened well-being of human kind [59]. Among the new pollutants, microplastics and nanoplastics have emerged as a cause of major concern which has negatively impacted terrestrial as well as aquatic ecosystems [60]. The worrisome aspect of the microplastic pollution is the fact that it nearly affects all the communities including microbes, plants, and animals residing in a particular ecosystem [61].

Figure 5.7 represents generation and circulation of microplastic pollutants into the environment.

The adversarial effects of microplastic pollution on terrestrial and aquatic ecosystem are described below:

5.2.3.1 Terrestrial Ecosystem

The rise in the usage of plastic products and their inefficient handling and improper disposals have led to a surge in microplastic pollution in the terrestrial ecosystem. Plastic products cover more than 10% of total waste generated globally, and due to their indestructible nature, majority of the waste ends up in landfills, waste piles, and agricultural fields [62]. Due to its persistent nature, plastic pollution is now considered a major cause of decline in biodiversity worldwide [63, 64]. The United Nations Environment Programme (UNEP) now lists microplastic pollution as one of the biggest environmental problems because of its hazardous effects on soil composition and nature as well as soil flora and fauna [65, 66].

The impact of aquatic microplastic pollution has been well studied, but the same is lacking for the terrestrial ecosystems. Extensive practices of soil waste landfills, dumps, and sludge fertilizers have caused huge microplastic pollution problem. One of the main sources of microplastics in soil is the use of sewage sludge for farming, plastic mulch aberration, and incidental plastic waste [67–69]. Moreover, constant settling of microplastic particles in soil occurs due to airborne transport, rainfall, industrial, and domestic discharges and atmospheric depositions [5]. The problem is so severe that microplastic particles have now been detected in the air as well due to

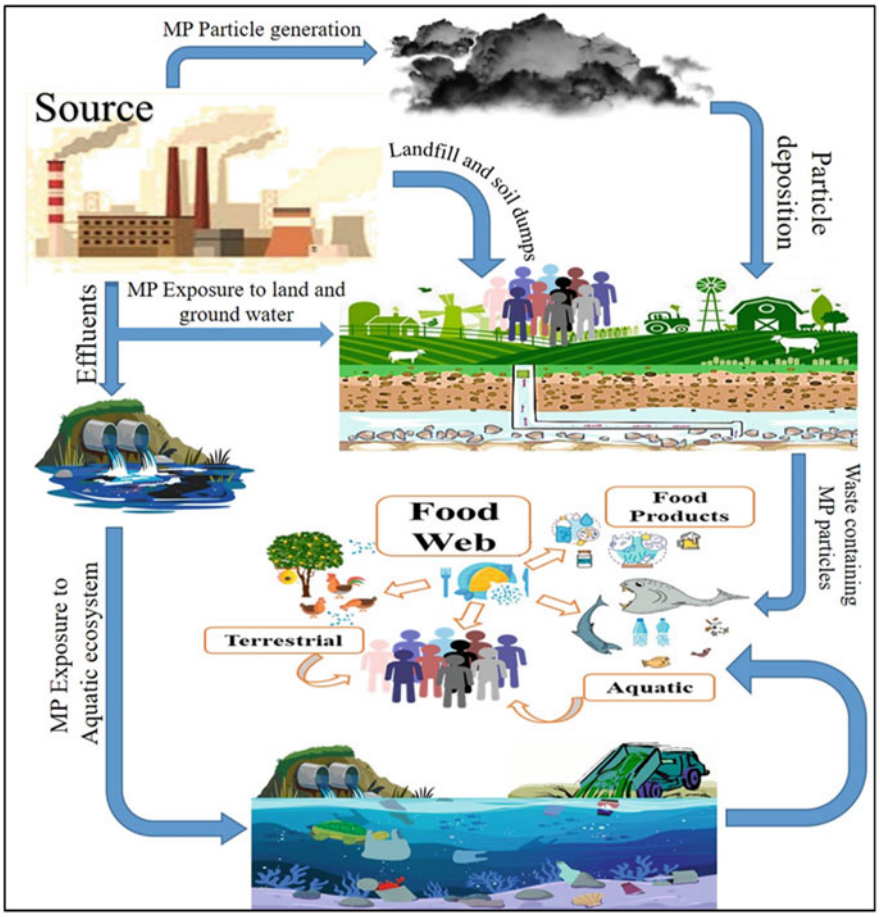

Fig. 5.7 Source and migration of microplastics into the environment

lack of efficient waste remediation [70]. It is suggested that high-density microplastics are more sensitive to wind and surface runoffs, thus more prone to surface depositions as compared to microplastic with low densities [33].

According to Horton et al. [6], terrestrial microplastic pollution problem is more serious than that of aquatic environment. The toxic effects of microplastics have started to appear in animal as well as plant species of terrestrial ecosystem [71]. The commonly found microplastic pollutants in soil contain micro- and nanoparticles, chemical additives, catalysts, residual monomers, and fillers [72, 73]. Apart from the toxicity of the aforementioned pollutants, they also serve as carriers for pathogens and absorb other chemical pollutants such as polychlorinated biphenyls, polyaromatic hydrocarbons, perfluoroalkyl substances, hexachlorocyclohexanes, dichlorodiphenyltrichloroethane, other pesticides, heavy metals, and pharmaceuticals. These contaminants often find their ways and constantly

accumulate in food web from where they might be consumed by secondary consumers [64, 74, 75]. The intentional or unintentional consumption of plastic particles along with food is a major factor behind microplastic toxicity in animals [76, 77].

Microplastics suppress the growth and reproduction, damage immune system, as well as alter gut microbiota of organisms including collembolan or springtails and earthworms [78–80]. It is reported that microplastic particles can trigger oxidative stress and damage the cells by producing ROS in some organism, such as *Brachionus koreanus* and *Dicentrarchus labrax* [81, 82]. The toxic effects of microplastics in soil organisms are induced by oxidative stress, internal organ damage, and impaired immune system [83]. The toxic effects of microplastics in mammals occur due to the accumulation in the liver, kidney, and digestive system [84, 85]. The accumulated microplastics in organs interact with microbiota by acting as a habitat for them. This results in the reduction in intestinal mucus secretion, damaging intestinal barrier and causing microbiota dysbiosis [86, 87]. Plastic polymers cause changes in biological characteristics (soil biota) as well as physico-chemical characteristics of soil structure such as bulk density, particle aggregation, hydrophobicity, and overall soil structure integrity. Thus, it adversely affects plants as well [88]. Microplastics accumulate in plants by water and nutrient absorption, thus causing delay in seed germination and adversely affecting the plant growth [89]. Jiang et al. [90] hypothesized that the toxicity in plants may be due to decrease in biomass generation, catalase activity, and growth retardation.

Some of the in vivo studies on terrestrial organisms for the toxic effects of micropollutants are compiled in Table 5.1.

There are very few studies that address the dangers microplastic pollutants pose to terrestrial plants and animals. To be more specific, the detailed genomic, proteomic, metabolomic, and toxicological studies are urgently called for unveiling the severity of damage that has already been caused or might be causing in the future by microplastic pollutants.

5.2.3.2 Effects on Aquatic Ecosystem

Microplastics are one of the most frequently reported pollutants that have been detected in the water bodies including ponds, lakes, rivers, as well as oceans. Microplastics can leach out from a wide range of products including fishing gears, fishing nets, boats and ships, packages, synthetic textiles, bottles, paints, bath gels, and polythene waste [3]. These plastic wastes often reach aquatic ecosystem and even constantly accumulate there. Some of the major sources for aquatic microplastic pollutants are industrial and urban effluents, beach sediment runoffs, oil and gas extraction fields, fishery waste, and litters released by anthropogenic sources [99]. Microplastic pollutants pose serious threat to aquatic animal health and the adverse effects have already started appearing [81].

Aquatic organisms such as planktons, crustaceans, worms, and fishes are directly (uptake of nutrition) or indirectly (predation) exposed to microplastics that are often found floating on water surfaces [100, 101]. These microplastics are so much in abundance that they can also enter into the bodies of aquatic organisms through

Table 5.1 Toxic effects of microplastics on terrestrial organisms

Terrestrial species	Microplastic pollutant	Toxic effects	References
Allium cepa	Bisphenol A	Chromosomal abnormalities and growth retardation	[91]
Aphylla williamsoni	Polyethylene	Changed neurophysiological functions	[92]
Mus musculus	Polyethylene	Behavioral changes (slower locomotion speed and higher anxiety index)	[93]
Eisenia fetida	Fluorescent polystyrene	Damaged intestinal cells, DNA damage	[94]
Lactuca sativa L.	Polystyrene	Accumulated in tissue	[95]
Vicia faba	Polystyrene fluorescent microplastics	Oxidative stress, inhibited growth	[96]
Lepidium sativum	Fluorescent polymer microspheres	Delayed germination and root growth	[89]
Folsomia candida	Polyethylene	Prompted avoidance behavior and repressed reproduction ability	[78]
Mus musculus	Fluorescent polystyrene	Change in gut microbiota, decreased intestinal mucus secretion, and compromised intestinal barrier function	[86]
Mus musculus	Polystyrene	Affect toxicity-based toxicokinetic/ toxicodynamic (TBTK/TD) behavior	[97]
Achatina fulica	PET fibers	Villi damages in walls of gastrointestinal tract	[83]
Triticum aestivum	LDPE, PET	Disturbed growth	[88]
Hypoaspis aculeifer, *Folsomia candida*	PVC	Change in physical properties of soil, inhibited growth and reproduction rate	[80]
Mus musculus	Polystyrene	Disruption to the microbiome in gut and lipid metabolism disorder in liver	[98]
Mus musculus	Fluorescent and pristine polystyrene microplastics	Accumulated in digestive system, oxidative stress, and neurotoxicity	[84]
Eisenia fetida	LDPE	Oxidative stress and changes in energy metabolism	[79]

water and agglomerate in their internal organs which can have detrimental effect in the physiological and biochemical functions [102]. The direct effect of microplastic particles and debris occurs by blockage of digestive and respiratory systems, which induces undernutrition, blocking nutrition flow, drowning suffocation, and even strangulation in aquatic animals [103]. Moreover, marine species such as sea snakes,

cetaceans, seals and sea lions, turtles, and various fishes are now endangered by direct entanglement and ingestion of plastic rings, fibers, tires, and debris. Some of the physiological manifestations which have become a common occurrence are scoliosis, skin aberration, tissue and organ damage, poor respiration, limited mobility, reproduction suppression, and undernourishment [104].

Microplastics are known to be toxic toward aquatic plants, especially phytoplankton occurring on surface water that may absorb or adsorb microplastic particles directly due to which photosynthetic activities as well as cell viabilities are compromised [105]. Similar to the planktons, microplastic particles and debris may also adhere to surfaces of microalgae which inhibit photosynthesis and thus affect growth and reproduction [106, 107]. Microplastic-contaminated microalgae have shown significant decrease in cell abundance and biomass because of restriction in energy and nutrient transfer, and therefore reduction in overall growth is observed [108, 109]. It may even lead to aggregation of particles in the algal cell wall followed by precipitation and physical damage to the algal surface [109]. Apart from this, large aquatic plants are also vulnerable to microplastic pollution due to direct hindrance of the root growth and blockage of nutrient uptake [35, 110]. In aquatic plants, microplastics cause oxidative stress that leads to increased reactive oxygen species (ROS) which in turn leads to lipid peroxidation, cell membrane damage, loss of selective permeability of membrane, destruction of cell membrane skeleton, and suppression of metabolic processes [111]. Toxic effects of various microplastic pollutants on aquatic species are listed in Table 5.2.

5.2.3.3 Effects on Humans

Plastic materials are indispensable part of everyday life, and their potential consequences toward human health have been greatly unexplored. Microplastics can have grave impact on human health because of their toxicity, persistent nature, and ability to carry other organic pollutants and heavy metals [120, 121]. There are different routes through which human beings can get exposed to plastic pollutants. According to Revel et al. [122], ingestion with food, inhalation with polluted air, and direct dermal contact are the major routes of microplastic pollutant exposure. According to an estimate, about 50,000 microplastic particles are consumed per person annually [123]. Many investigations have revealed that microplastic pollutants were mainly composed of PP, PE, PS, and PET. The exposure of PE to human beings is generally from plastic films used in materials like polythene bags as well as food packaging materials [124]. Moreover, humans are often exposed to PS from plastic packaging and manufacturing industries, while PP and PET microplastics are commonly released from polyester fabric, textiles, and reusable products [125]. Apart from this, humans may get exposed to plastic pollutants from the combustion of large plastic pieces, landfill waste disposal, industrial effluents and discharges, and dust redeposition [70].

According to Prata [126], microplastic particles that are inhaled and ingested are likely to be subjected to body's natural defense and clearance mechanisms such as phagocytosis, sneezing, mucociliary escalator, and lymphatic transport and excretion. However, some particles may resist clearance and may reach to the respiratory

Table 5.2 Impact of microplastics on aquatic organisms

Aquatic species	Microplastic pollutant	Toxic effects	References
Danio rerio	Microplastic particles	Increase in apoptotic and necrotic erythrocytes	[112]
Paracentrotus lividus	PB, MG, and a "virgin" PE resin	Cytotoxic effect (lethal)	[113]
Utricularia vulgaris	PS particles	Leaf growth functionality impaired, oxidative damage observed	[114]
Artemia nauplii Daphnia magna	Plastic foam (PS)	Acute toxicity	[115]
Skeletonema grethae Dunaliella tertiolecta Thalassiosira pseudonana	PS particles	Modifications in EPS composition and protein aggregate formation	[116]
Spirodela polyrhiza S. polyrhiza	PS particles	MPs absorption, bioaccumulation, and biomagnification	[107]
Lemna minor	PE particles	The root growth inhibited	[110]
Carassius auratus	Microplastic debris	Accumulate in the gut and negative effect on nutrition	[14]
Chaetoceros neogracile Tisochrysis lutea	PS particles	Bioaccumulation in algal biomass which affected nutrition	[117, 118]
Dunaliella tertiolecta Thalassiosira pseudonana	PS particles	Cell mass and size decrease due to growth inhibition	[119]
Hyalella azteca	Microplastic particles	Growth rate and reproduction rate impaired	[52]

and digestive system. It may lead to release of cellular messenger molecules, various proteases, and reactive oxygen species (ROS) which can trigger inflammatory response in the body [5, 127]. Law et al. [128] reported that polymer fibers can persist in a simulated lung fluid system for about 6 months without any changes in their surface morphology, which indicates that microplastic particles can agglomerate and retain in the human lungs for longer period of time and may induce cell damage due to constant generation of ROS. Although the investigations on detrimental consequences of microplastic exposure are limited, occupational diseases with respiratory symptoms, such as breathing problem, throat irritation, cough, and chest pain in plastic industry workers, are well documented [129]. Additionally, chronic inflammation and irritation may eventually cause cancer resulting from lung fibrosis [122, 130]. In addition to plastics, plastic additives such as bisphenol A (BPA) also induce oxidative damage in human erythrocytes, L-02 hepatocytes, and

placental and endometrial stromal cells [131]. BPA exposure eventually upregulates expression of inflammatory cytokines and estrogen receptor ER-α which may result in endocrine system-related diseases [132].

Nearly every ecosystem and all organisms associated with it are being exposed to plastic pollutants that reach into various body organs via air, water, food, or personal care products and accumulate there. This has threatened the biodiversity and humanity as well. Thus, remediation of these plastic pollutants is an urgent need to protect every living being on the planet.

5.2.4 Remediation of Microplastics

Among the different chemical pollutants, microplastics pose more threat to the living beings due to its higher resistance against physical, chemical, and biological agents, ability to accumulate, and ability to carry other pollutants as well as pathogens. As mentioned above, it is an inherent property of plastic material to resist against various acids, alkalis, organic solvents, and oxidative agents.

Due to such high resistance power, degradation and disposal of plastic materials have become very challenging tasks, and their improper discarding has often led to plastic pollution [133]. Among the plastic degradation strategies, biological mode of degradation has been a subject of great interest recently because of its higher efficiency and environmentally benign nature [134]. It is well-known that microbes have ability to break down polymers into monomeric organic residues and mineralize as well as redistribute them into C, N, S, and P elementary cycles [135].

5.2.4.1 Microbes' Microplastic Bioremediation

Eukaryotic fungi and prokaryotic bacteria have obtained great attention for bioremediation of xenobiotic as well as natural polymers [136]. Tokiwa et al. [137] have briefly explained the biological mechanism of microbial plastic degradation. Initially, microbes attach and colonize on the surface of plastic materials, which is followed by catalytic degradation. The enzymes bind with plastic material and continuously cleave it by hydrolysis which causes the breakdown of polymers into low molecular weight oligomers and eventually then to dimers and finally into monomeric units. This process continues till mineralization and eventual liberation of CO_2 and H_2O. The rate of biological degradation is influenced by plastic biodegradability, enzyme type, physicochemical properties of plastic polymer, tensile properties, extent of fragmentation, functional groups, and crystallinity [115]. The more the crystallinity, the more it will be difficult to degrade for the microbes, and thus the amorphous region is degraded first which ultimately opens up polymer structure for further degradation [16]. Furthermore, pretreatments of microplastic can improve the degradation potential by enhancing the susceptibility and reducing the hydrophobicity of microplastics [24]. In recent times, isolating the microbes capable of microplastic degradation have been prioritized from polluted terrestrial and aquatic environments as they may already possess adapted metabolic processes for it [138].

Several bacterial species are able to catalyze metabolic reactions that are involved in microplastic adsorption and breakdown [139, 140]. These microorganisms may use polymer material as a source of carbon in minimal nutrient media; thus, it can reduce dry weight and change molecular structure which may eventually induce morphological alterations in polymer structure [141]. According to Yuan et al. [142], use of bacterial consortia has better prospective of plastic degradation because a stable microbial community can perform more efficiently and simultaneously eliminate the probable side products or toxic metabolites produced by other strains. However, the overall efficiency of bacterial system toward plastic degradation is less than that of fungal system [143].

5.2.4.2 Fungi: A Potent Biological System for Microplastic Remediation

Fungi possess a wide range of metabolic processes and have ability to synthesize various extracellular multienzyme complexes as well as important secondary metabolites which make them the most suitable candidates to be investigated for their microplastic biodegradation capabilities [15, 47]. Additionally, fungi can decrease the hydrophobicity of plastics by means of introducing various chemical bonds such as carbonyl, carboxyl, and ester which in turn make plastic materials even more vulnerable to degradation. Many fungal species have been popular to exploit plastic as a lone nutrient source [144, 145] under natural (soil and compost piles) as well as artificial (solid-state and submerged) conditions, which proves that fungi are the most versatile and proficient organisms for plastic bioremediation [146, 147].

All classes of fungi including ascomycetes, basidiomycetes, and zygomycetes have been reported for degradation of plastic compounds such as polyethylene terephthalate (PET), PVC, PP, polyesters, and microplastic particles. The efficiency of fungal species to degrade plastics can be assessed by evaluating the biomass growth and/or changes occurring in the polymer structure and chemistry. Some of the other biochemical tests such as biochemical oxygen demand (BOD), enzyme assays, and assessment of byproducts as well as gases (CO_2) can give more information regarding the overall potential of isolates. Several strains of *Aspergillus*, *Penicillium*, *Mucor*, and *Trichoderma* have been commonly reported as the microplastic degrading fungi [16, 143, 148]. Some of the important discoveries of plastic degrading fungal isolates are compiled in Table 5.3.

Polyethylene (PE) is one of the most copiously occurring microplastics in the nature. Sowmya et al. [163] isolated *Trichoderma harzianum* that was able to degrade UV radiation-treated PE up to 40%. In one of the important studies, a marine fungus *Zalerion maritimum* used PE as a lone carbon source and displayed highest degradation (43%) capability [13]. Zhang et al. [171] isolated fungal isolate *Aspergillus flavus* PEDX3 from the gastrointestinal tract of *Galleria mellonella*—a wax moth—which was capable of PE degradation. Upon incubation of 28 days, this isolate carried out degradation of HDPE microplastic and converted them into low molecular weight compounds. Here the PE degradation was validated using Fourier transform-infrared spectroscopy (FT-IR) by checking the presence of carbonyl and ether groups. Spina et al. [172] studied the fungal community of a landfill soil

Table 5.3 Summary of fungal species capable of microplastic remediation

Strains	Plastic polymer	Major observations	References
Apiotrichum porosum, *Penicillium samsonianum*, *Talaromyces pinophilus*, *Purpureocillium lilacinum*	PE	Halos indicating potential plastic degradation after 7 days	[148]
Penicillium, *Geomyces*, *Mortierella* species	PU, PS, and PE	Highest degradation of polyurethane (28.34%) detected after 3 months Detection of PU degradation via FTIR	[149]
Aspergillus Niger, *Aspergillus flavus*, and *Aspergillus oryzae*	PE	Maximum of 26.15% weight loss after 55 days SEM and FTIR data showed degradation	[150]
Cephalosporium sp. *NCIM 1251*	PS	20.62 ± 1.47% weight loss detected after 2 months	[151]
Mucor sp. *(NCIM 881)*	PS	Degradation detected in FTIR spectra after 60 days	[151]
Pleurotus ostreatus	PE	SCA was decreased by 84%, degradation detected in FTIR spectra after 60 days	[152]
Penicillium sp.	PU	Visual signs of degradation observed through SEM analysis, detection of PU degradation via FTIR after 2 months	[153]
Cladosporium cladosporioides Xepiculopsis graminea Penicillium griseofulvum	PU	Halos around the growth—sign of plastic degradation	[154]
Trichoderma viride	LDPE	5.13% weight loss and 58% reduction in tensile strength after 45 days	[155]
Aspergillus tubingensis	PU	Cracking of surface, micro-poration, and erosion were observed through SEM, ATR-FTIR detected degradation products after 20 days	[156]
Penicillium oxalicum	LDPE and HDPE sheets	Visual signs of degradation observed through SEM analysis Detection of PU degradation via FTIR after 2 months	[157]
Penicillium sp.	PE	PE weight loss—43.4%	[158]
Zalerion maritimum (ATTC 34329)	PE	56.7 ± 2.9% mass loss was observed	[13]
	PU (Impranil)	GCMS and FTIR confirmed degradation of Impranil (PU)	[159]

(continued)

Table 5.3 (continued)

Strains	Plastic polymer	Major observations	References
Cladosporium pseudocladosporioides strain T1.PL.1			
Aspergillus tubingensis VRKPT1	HDPE	6 ± 0.2% weight loss after 30 days	[160]
Eupenicillium hirayamae	LDPE	Increased biomass production and production of MnP and laccase after 28 days	[11]
Gloeophyllum trabeum	PS (polystyrene sulfonate)	Up to 50% depolymerization of PS after 20 days	[161]
Lasiodiplodia theobromae	LDPE and PP	Laccase production, weight loss in gamma-irradiated PP and PE after 90 days with sign of degradation products via FTIR	[162]
Paecilomyces variotii	LDPE	Increased biomass production and production of MnP, LiP, and laccase after 28 days	[11]
Penicillium simplicissimum	PE	38% weight loss for UV-treated polyethylene FTIR and NMR confirmed degradation of PE Simultaneous production of laccase and MnP was observed	[163]
Phialophora alba	LDPE	Increased biomass production and production of MnP and laccase after 28 days	[11]
Trichoderma harzianum	PE	40% weight loss of UV PE. Laccase and MnP mediated degradation	[163]
Aspergillus terreus MF12	HDPE	Highest degradation rates for UV-treated PE, i.e., within 30 days	[164]
Fusarium sp.	Nylon-4	Reduction in average weight	[165]
Thermomyces Fusarium solani	PET (low crystallinity)	Cutinase-mediated degradation of PET into TPA with 97% weight loss	[166]
Bjerkandera adusta	Nylon-6	Decrease MnP production in the presence of nylon	[167]
Fusarium oxysporum	PET	Increment in hydrophobicity and release of TPA	[168]
Penicillium citrinum	PET	Polyesterase-mediated hydrolysis of PET showed by	[19]
Penicillium simplicissimum YK	PE	Reduction in molecular weight	[145]
Strain IZU-154	PE	Production of MnP and decrease in tensile strength	[169]

(continued)

Table 5.3 (continued)

Strains	Plastic polymer	Major observations	References
Strain IZU-154	Nylon-6	NMR studies showed formation of four end groups, CHO, NHCHO, CH_3, and $CONH_2$, which are indicators of degradation	[170]

contaminated with plastic waste and isolated 95 potential PE utilizing fungi belonging to *Ascomycota* phylum. About 97% of the isolates had ability to grow by utilizing PE powder (0.5–1%) as sole source of carbon. Among these isolates, the species from *Fusarium* genera had higher tolerance toward PE. *Fusarium falciforme*, *Purpureocillium lilacinum*, and *Fusarium oxysporum* showed maximum PE degradation mediated through strong oxidation reactions and physical damage in the PE film. In a novel study, Oviedo-Anchundia et al. [149] isolated *Penicillium*, *Geomyces*, and *Mortierella* species from Antarctica, which were able to degrade PE materials.

Polystyrene (PS) degradation has been problematic for researchers as it is nonrecyclable and its biodegradability is very poor as evident with studies in *Cephalosporium* sp. and *Mucor* sp. [151]. Castiglia and Kuhar [173] reported that fungus *Aureobasidium pullulans* has ability to degrade PS. Moreover, Yanto et al. [174] reported improved biodegradation of PS with optimized media containing malt extract liquid which promoted microbial growth along with enzyme production, thus enhancing the overall PS degradation. Fungal strains of *Penicillium* and *Geomyces* genera degraded PS by 8.39% and 6.82%, respectively, whereas *Mortierella* spp. was able to degrade PS material up to 2.19% [149].

The insights on fungal degradation of polyvinylchloride (PVC) are not yet clearly revealed as it has not been studied by many researchers; but there are evidences for the fungal degradation of PVC plastic materials [175]. Ali et al. [176] reported *Phanerochaete chrysosporium* can adhere and grow on PVC film which proves its potential for degradation. Sumathi et al. [177] isolated *Cochliobolus* sp.—a fungal species—from plastic-contaminated soils and cultured it with commercially available low molecular weight polyvinyl chloride (PVC) which revealed morphological and molecular changes in the polymer structure. Vivi et al. [62] observed 75% of mass loss in unplasticized PVC films during a study involving use of *Penicillium funiculosum*, *Aspergillus brasiliensis*, *Paecilomyces variotii*, and *Trichoderma virens* upon 28 days of treatment.

Polyurethane (PU) has been reported as easy to degrade at normal laboratory conditions and it has been evident in many studies [144, 178]. Many fungal species such as *Nectria* species, *Plectosphaerella*, *Geomyces pannorum*, *Penicillium inflatum*, *Neonectria ramulariae*, *Penicillium venetum*, and *Penicillium viridicatum* have potential to effectively remediate PU materials [179]. Osman et al. [180] isolated soil fungi *Aspergillus* sp. strain S45 which showed weight loss, evolution

of CO_2, and breakdown of PU film. Oviedo-Anchundia et al. [149] reported that *Penicillium* spp. displayed maximum 28.34% of PU film degradation in 3 months.

5.2.4.3 Metabolic Pathways Involved in Microplastic Remediation

Biological remediation of plastics is an eco-friendly approach. Microbes can modify, transform, and degrade plastic materials through various catalytic reactions [181]. Although the complete mechanism of plastic remediation through microbial metabolism is not fully understood, still some of the hypothetical biodegradation pathways have been reported [10, 182].

PE plastics and their chemical derivatives such as polylactic acid (PLA) are easily biodegradable as compared to other plastics [183]. According to Oliveira et al. [1], the degradation of PE and PLA begins by depolymerization and conversion of polymer matrix into oligomers (Fig. 5.8). By the action of hydrolase and esterase enzymes, ester bonds are cleaved and as a result, carboxyl and hydroxyl groups are

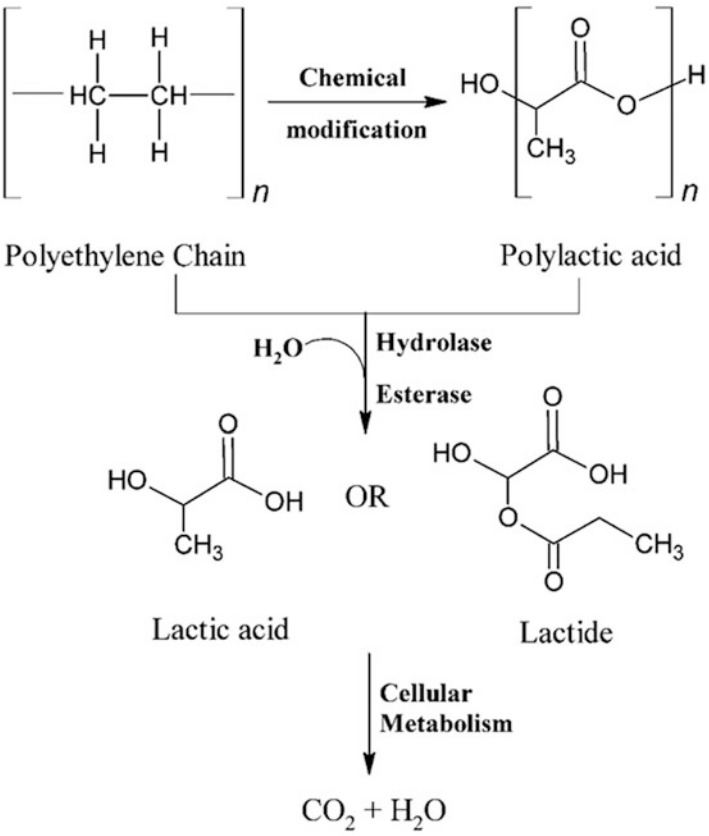

Fig. 5.8 Microbial degradation pathway of PE plastic [1]

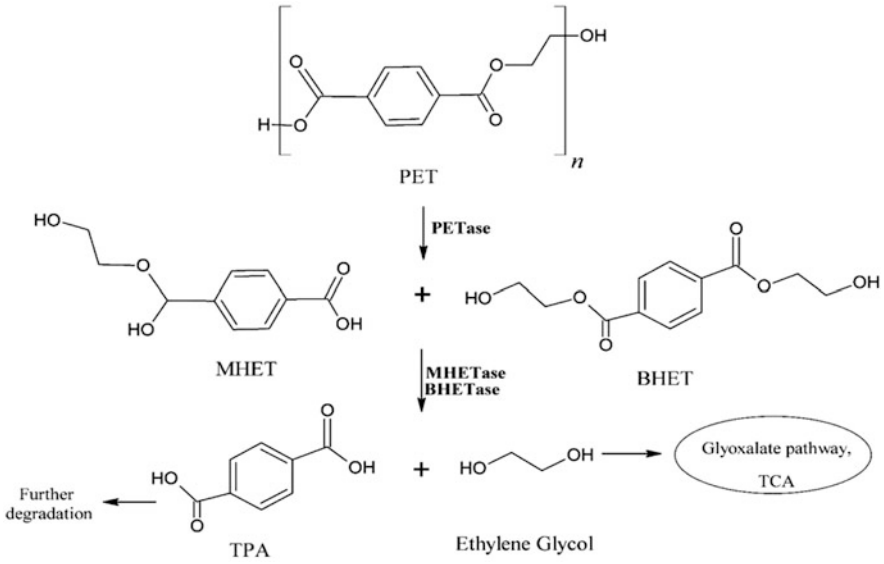

Fig. 5.9 Microbial remediation pathway of PET plastic

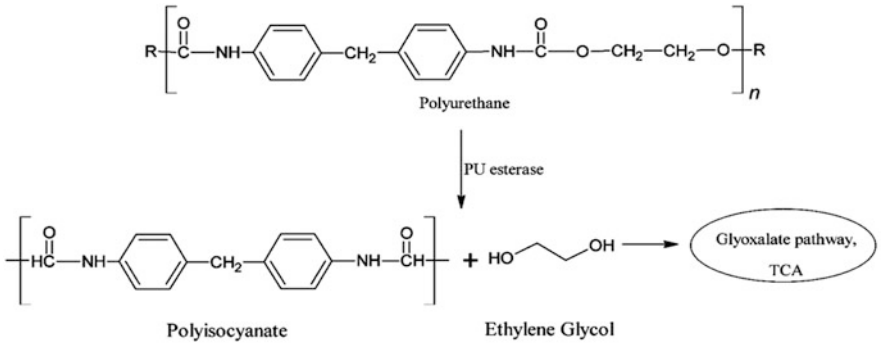

Fig. 5.10 Microbe-mediated PU plastic degradation pathway [188]

exposed [184]. This leads to generation of simple organic molecules such as lactic acid and lactides, which can be easily utilized by fungi by cellular metabolism [185].

Microorganisms especially fungi have capabilities to degrade PET plastics by means of enzyme PETase (Fig. 5.9). This enzyme is extracellular in nature and it hydrolyzes PET into monomeric mono-2-hydroxyethyl terephthalate (MHET) and bis(2-hydroxyethyl)-TPA (BHET). Such products are further degraded by MHETase and BHETase enzymes to form simple terephthalic acid (TPA) and ethylene glycol [182]. These simple compounds can directly undergo fungal metabolic pathways including glyoxylate pathway and tricarboxylic acid (TCA) cycle.

PU is a biodegradable plastic which can be generally hydrolyzed through hydrolytic cleavage of urethane bonds [186, 187]. As depicted in Fig. 5.10, PU esterase

cleaves PU into polyisocyanate and ethylene glycol. Ethylene glycol is easily taken up and converted into glycolic acid by fungi through their cellular metabolism [188]. Polyisocyanate is also degraded into simple monomers but the pathway has not been studied yet.

5.3 Conclusions and Future Perspectives

Plastics are one of the most abundantly manufactured xenobiotics around the globe due their wide variety of application in nearly every single industrial sector as well as domestic purpose. As a result, no current recycling and waste management systems are capable enough to contain and treat microplastic waste that is generated afterward. Over the past decade, human reliance on plastic has been increasing, and due to this, microplastic pollution has reached into every ecosystems and niches of earth. What makes this issue most urgent is the fact that these pollutants can last for years and magnify and already have started causing health hazards in all living beings including humans. Thus, it is the need of the hour that researchers around the world come up with long-term sustainable solution to this problem. In our literature review, we found that fungal remediation strategies have better potential to tackle this problem over physicochemical methods as they are less effective with microplastic compound that persists in the environment. This chapter emphasizes on the types of plastic pollutants and their hazardous effect on various ecosystems and reviewing potential mechanism of fungal remediation of microplastic pollutants. Fungi possess an array of enzymes that can interact with plastic polymer which otherwise are difficult to degrade due to their hydrophobicity and resistance to physical factors (temperature, photooxidation, and chemicals).

Although the ability of diverse fungal species to degrade and potentially mineralize plastics like HDPE, LDPE, PU, PET, and PE is evident from the bioremediation studies, more research is required to understand exact enzymatic mechanisms and role of undiscovered metabolites. There have been little success and evidences regarding tackling PP and PVC microplastics. The mycoremediation can benefit greatly by employing genomic, proteomic, and metabolomic approaches as it hasn't been attempted much yet. Furthermore, a more practical approach is required for field trials before recommending fungal bioremediation as a tangible solution for microplastics.

References

1. Oliveira J, Belchior A et al (2020) Marine environmental plastic pollution: mitigation by microorganism degradation and recycling valorization. Front Mar Sci 7
2. Hosler D, Burkett SL, Tarkanian MJ (1999) Prehistoric polymers: rubber processing in ancient Mesoamerica. Science 284(5422):1988–1991
3. Andrady AL (2011) Microplastics in the marine environment. Mar Pollut Bull 62(8): 1596–1605

4. Essel EE, Nematollahi A et al (2015) Effects of upstream roughness and Reynolds number on separated and reattached turbulent flow. J Turbul 16(9):872–899
5. Gasperi J, Wright SL et al (2018) Microplastics in air: are we breathing it in? Curr Opin Environ Sci Health 1:1–5
6. Horton AA, Walton A et al (2017) Microplastics in freshwater and terrestrial environments: evaluating the current understanding to identify the knowledge gaps and future research priorities. Sci Total Environ 586:127–141
7. Geyer R, Jambeck JR, Law KL (2017) Producción, uso y destino de todos los plásticos jamás fabricados. Sci Adv 3(7):1207–1221
8. Horton AA, Barnes DK (2020) Microplastic pollution in a rapidly changing world: implications for remote and vulnerable marine ecosystems. Sci Total Environ 738:140349
9. Cowan AR, Costanzo CM et al (2022) Fungal bioremediation of polyethylene: challenges and perspectives. J Appl Microbiol 132(1):78–89
10. Sánchez C (2020) Fungal potential for the degradation of petroleum-based polymers: an overview of macro-and microplastics biodegradation. Biotechnol Adv 40:107501
11. Ameen F, Moslem M et al (2015) Biodegradation of low density polyethylene (LDPE) by mangrove fungi from the red sea coast. Prog Rubber Plast Recycl Technol 31(2):125–143
12. Krueger MC, Harms H, Schlosser D (2015) Prospects for microbiological solutions to environmental pollution with plastics. Appl Microbiol Biotechnol 99(21):8857–8874
13. Paço A, Duarte K et al (2017) Biodegradation of polyethylene microplastics by the marine fungus Zalerion maritimum. Sci Total Environ 586:10–15
14. Grigorakis S, Mason SA, Drouillard KG (2017) Determination of the gut retention of plastic microbeads and microfibers in goldfish (*Carassius auratus*). Chemosphere 169:233–238
15. Kawai F, Kawabata T, Oda M (2019) Current knowledge on enzymatic PET degradation and its possible application to waste stream management and other fields. Appl Microbiol Biotechnol 103(11):4253–4268
16. Restrepo-Flórez JM, Bassi A, Thompson MR (2014) Microbial degradation and deterioration of polyethylene–a review. Int Biodeter Biodegrad 88:83–90
17. El-Morsy EM, Hassan HM, Ahmed E (2017) Biodegradative activities of fungal isolates from plastic contaminated soils. Mycosphere 8(8):1071–1087
18. Danso D, Chow J and Streit WR (2019) Plastics: microbial degradation, environmental and biotechnological perspectives. Appl Environ Microbiol
19. Liebminger S, Eberl A et al (2007) Hydrolysis of PET and bis-(benzoyloxyethyl) terephthalate with a new polyesterase from *Penicillium citrinum*. Biocatal Biotransformation 25(2–4): 171–177
20. Ghatge S, Yang Y et al (2020) Biodegradation of polyethylene: a brief review. Appl Biol Chem 63(1):1–14
21. Montazer Z, Habibi-Najafi MB, Levin DB (2020) Challenges with verifying microbial degradation of polyethylene. Polymers 12(1):123
22. Bhatt P, Pathak VM et al (2021) Microplastic contaminants in the aqueous environment, fate, toxicity consequences, and remediation strategies. Environ Res 200:111762
23. Law KL, Thompson RC (2014) Microplastics in the seas. Science 345(6193):144–145
24. Arutchelvi J, Sudhakar M et al (2008) Biodegradation of polyethylene and polypropylene. Indian J Biotechnol 7:9–22
25. Bardají DKR, Moretto JAS et al (2020) A mini-review: current advances in polyethylene biodegradation. World J Microbiol Biotechnol 36(2):1–10
26. Klyosov AA (2007) Wood-plastic composites. John Wiley & Sons
27. https://www.calpaclab.com/chemical-compatibility-charts
28. Scalenghe R (2018) Resource or waste? A perspective of plastics degradation in soil with a focus on end-of-life options. Heliyon 4(12):e00941
29. Trinh NH (2019) Development of palm kernel oil polyol-based shape memory polyurethane with polyethylene glycol (peg) and poly caprolactone (pcl) as soft segment. Mater Res Express 7(2):025704
30. Wünsch JR (2000) The economic significance of polystyrene. In: Polystyrene: synthesis, production and applications, vol 1, pp 1–5

31. Domininghaus H (2005) Synthetische Kunststoffe. Die Kunststoffe und ihre Eigenschaften:451–1442
32. Mor R, Sivan A (2008) Biofilm formation and partial biodegradation of polystyrene by the actinomycete Rhodococcus ruber. Biodegradation 19(6):851–858
33. Du F, Cai H et al (2020) Microplastics in take-out food containers. J Hazard Mater 399:122969
34. World Health Organization (2016) Physical activity strategy for the WHO European Region 2016–2025
35. Verbeek CJ & Gavin C (2020) Grafting functional groups onto biodegradable thermoplastic polyesters. In Reactive and functional polymers Volume Two. Springer, Cham. pp. 245–281
36. Verbeek JH, Rajamaki B et al (2020) Personal protective equipment for preventing highly infectious diseases due to exposure to contaminated body fluids in healthcare staff. Cochrane Database Syst Rev 4:1–147
37. Crawford CB & Quinn B (2017) Physiochemical properties and degradation. In: Microplastic pollutants. Elsevier, pp. 57–100
38. de Sousa FDB (2021) Plastic and its consequences during the COVID-19 pandemic. Environ Sci Pollut Res:1–12
39. Harussani MM, Sapuan SM et al (2022) Pyrolysis of polypropylene plastic waste into carbonaceous char: priority of plastic waste management amidst COVID-19 pandemic. Sci Total Environ 803:149911
40. Peng BY, Chen Z et al (2020) Biodegradation of polyvinyl chloride (PVC) in Tenebrio molitor (Coleoptera: Tenebrionidae) larvae. Environ Int 145:106106
41. Ceresana (2014) Global demand for PVC to rise by about 3.2%/year to 2021. Addit Polym 2014(11):10–11
42. https://www.plasticseurope.org/application/files/1115/7236/4388/FINAL_web_version_Plastics_the_facts2019_14102019.pdf (accessed May 01, 2020)
43. Close LG, Gilbert RD, Fornes RE (1977) Poly (vinyl chloride) degradation—a review. Polym-Plast Tech Mat 8(2):177–198
44. Akindoyo JO, Beg MD et al (2016) Polyurethane types, synthesis and applications–a review. RSC Adv 6(115):114453–114482
45. Heath DE, Guelcher SA and Cooper SL (2020) Polyurethanes. In Biomaterials science. Academic Press. pp. 103–107
46. Singh H, Jain AK (2008) Ignition, combustion, toxicity, and fire retardancy of polyurethane foams: a comprehensive review. J Appl Polym Sci 111:1115–1143
47. Marturano V, Cerruti P, Ambrogi V (2017) Polymer additives. Phys Sci Rev 6:20160130
48. Crompton TR (2007) Additive migration from plastics into foods: a guide for analytical chemists. Smithers Rapra Publishing
49. Hahladakis JN, Velis CA et al (2018) An overview of chemical additives present in plastics: migration, release, fate and environmental impact during their use, disposal and recycling. J Hazard Mater 344:179–199
50. Burgos-Aceves MA, Abo-Al-Ela HG and Faggio C (2021) Impact of phthalates and bisphenols plasticizers on haemocyte immune function of aquatic invertebrates: a review on physiological, biochemical, and genomic aspects. J Hazard Mater 126426
51. Bhunia K, Sablani SS et al (2013) Migration of chemical compounds from packaging polymers during microwave, conventional heat treatment, and storage. Compr Rev Food Sci Food Safe 12(5):523–545
52. Burgos-Aceves MA, Abo-Al-Ela HG, Faggio C (2021) Physiological and metabolic approach of plastic additive effects: immune cells responses. J Hazard Mater 404:124114
53. Ambrogi V, Carfagna C et al. (2017) Additives in polymers. In Modification of polymer properties. William Andrew Publishing. pp. 87–108
54. Morgan AB, Gilman JW (2013) An overview of flame retardancy of polymeric materials: application, technology, and future directions. Fire Mat 37(4):259–279

55. Muir DC, de Wit CA (2010) Trends of legacy and new persistent organic pollutants in the circumpolar arctic: overview, conclusions, and recommendations. Sci Total Environ 408(15): 3044–3051
56. Höfer R (2012) Processing and performance additives for plastics. In: Polymer science: a comprehensive reference – polymers for a sustainable environment and green energy, vol 1, pp 369–381
57. Lau OW, Wong SK (2000) Contamination in food from packaging material. J Chromatogr A 882(1–2):255–270
58. Hermabessiere L, Dehaut A et al (2017) Occurrence and effects of plastic additives on marine environments and organisms: a review. Chemosphere 182:781–793
59. Christensen CM (2006) The ongoing process of building a theory of disruption. J Product Innovat Manag 23(1):39–55
60. Zhang F, Xu J et al (2021) Seasonal distributions of microplastics and estimation of the microplastic load ingested by wild caught fish in the East China Sea. J Hazard Mater 419: 126456
61. Ge J, Li H et al. (2021) Review of the toxic effect of microplastics on terrestrial and aquatic plants. Sci Total Environ. p.148333
62. Wang MH, He Y, Sen B (2019) Research and management of plastic pollution in coastal environments of China. Environ Pollut 248:898–905
63. Barnes DK, Galgani F et al (2009) Accumulation and fragmentation of plastic debris in global environments. Philos Trans R Soc B Biol Sci 364(1526):1985–1998
64. Wang W, Ge J et al (2020) Environmental fate and impacts of microplastics in soil ecosystems: Progress and perspective. Sci Total Environ 708:134841
65. Scheurer M, Bigalke M (2018) Microplastics in Swiss floodplain soils. Environ Sci Technol 52(6):3591–3598
66. UNEP, UNEP frontiers 2016 report: Emerging issues of environmental concern; Nairobi, 2016
67. Farmer J, Zhang B et al (2017) Long-term effect of plastic film mulching and fertilization on bacterial communities in a brown soil revealed by high through-put sequencing. Arch Agron Soil Sci 63(2):230–241
68. Nizzetto L, Bussi G et al (2016) A theoretical assessment of microplastic transport in river catchments and their retention by soils and river sediments. Environ Sci Process Impacts 18(8): 1050–1059
69. Ziajahromi S, Neale PA et al (2017) Wastewater treatment plants as a pathway for microplastics: development of a new approach to sample wastewater-based microplastics. Water Res 112:93–99
70. Dris R, Gasperi J et al (2017) A first overview of textile fibers, including microplastics, in indoor and outdoor environments. Environ Pollut 221:453–458
71. Rillig MC (2012) Microplastic in terrestrial ecosystems and the soil? Environ Sci Technol 46(12):6453–6454
72. Fendall LS, Sewell MA (2009) Contributing to marine pollution by washing your face: microplastics in facial cleansers. Mar Pollut Bull 58(8):1225–1228
73. Rochman CM, Hoh E et al (2013) Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. Sci Rep 3(1):1–7
74. Besseling E, Quik JT et al (2017) Fate of nano- and microplastic in freshwater systems: a modeling study. Environ Pollut 220:540–548
75. Hodson ME, Duffus-Hodson CA et al (2017) Plastic bag derived-microplastics as a vector for metal exposure in terrestrial invertebrates. Environ Sci Technol 51(8):4714–4721
76. Rillig MC, Ziersch L, Hempel S (2017) Microplastic transport in soil by earthworms. Sci Rep 7(1):1–6
77. Zhu D, Bi QF et al (2018) Trophic predator-prey relationships promote transport of microplastics compared with the single Hypoaspis aculeifer and Folsomia candida. Environ Pollut 235:150–154
78. Ju H, Zhu D, Qiao M (2019) Effects of polyethylene microplastics on the gut microbial community, reproduction and avoidance behaviors of the soil springtail, Folsomia candida. Environ Pollut 247:890–897

79. Rodriguez-Seijo A, Lourenço J et al (2017) Histopathological and molecular effects of microplastics in Eisenia andrei Bouché. Environ Pollut 220:495–503
80. Zhu D, Chen QL et al (2018) Exposure of soil collembolans to microplastics perturbs their gut microbiota and alters their isotopic composition. Soil Biol Biochem 116:302–310
81. Barboza LGA, Vethaak AD et al (2018) Marine microplastic debris: an emerging issue for food security, food safety and human health. Mar Pollut Bull 133:336–348
82. Jeong CB, Won EJ et al (2016) Microplastic size-dependent toxicity, oxidative stress induction, and p-JNK and p-p38 activation in the monogonont rotifer (*Brachionus koreanus*). Environ Sci Technol 50(16):8849–8857
83. Song Y, Cao C et al (2019) Uptake and adverse effects of polyethylene terephthalate microplastics fibers on terrestrial snails (Achatina fulica) after soil exposure. Environ Pollut 250:447–455
84. Deng Y, Zhang Y et al (2017) Tissue accumulation of microplastics in mice and biomarker responses suggest widespread health risks of exposure. Sci Rep 7(1):1–10
85. Yang L, Li K et al (2019) Removal of microplastics in municipal sewage from China's largest water reclamation plant. Water Res 155:175–181
86. Jin Y, Lu L et al (2019) Impacts of polystyrene microplastic on the gut barrier, microbiota and metabolism of mice. Sci Total Environ 649:308–317
87. Lu L, Wan Z et al (2018) Polystyrene microplastics induce gut microbiota dysbiosis and hepatic lipid metabolism disorder in mice. Sci Total Environ 631:449–458
88. Qi Y, Yang X et al (2018) Macro-and micro-plastics in soil-plant system: effects of plastic mulch film residues on wheat (Triticum aestivum) growth. Sci Total Environ 645:1048–1056
89. Bosker T, Bouwman LJ et al (2019) Microplastics accumulate on pores in seed capsule and delay germination and root growth of the terrestrial vascular plant *Lepidium sativum*. Chemosphere 226:774–781
90. Jiang C, Yin L et al (2019) Microplastic pollution in the rivers of the Tibet plateau. Environ Pollut 249:91–98
91. Trivedi J, Chhaya U (2021) Bioremediation of phenolic pollutant bisphenol A using optimized reverse micelles system of Trametes versicolor laccase in non-aqueous environment. 3 Biotech 11(6):1–14
92. Chagas TQ, da Costa Araújo AP, Malafaia G (2021) Biomicroplastics versus conventional microplastics: an insight on the toxicity of these polymers in dragonfly larvae. Sci Total Environ 761:143231
93. da Costa Araújo AP, Malafaia G (2021) Microplastic ingestion induces behavioral disorders in mice: a preliminary study on the trophic transfer effects via tadpoles and fish. J Hazard Mater 401:123263
94. Jiang X, Chang Y et al (2020) Toxicological effects of polystyrene microplastics on earthworm (*Eisenia fetida*). Environ Pollut 259:113896
95. Li Z, Li Q et al (2020) Physiological responses of lettuce (*Lactuca* sativa L.) to microplastic pollution. Environ Sci Pollut Res 27:30306–30314
96. Jiang X, Chen H et al (2019) Ecotoxicity and genotoxicity of polystyrene microplastics on higher plant *Vicia faba*. Environ Pollut 250:831–838
97. Yang H, Yang T et al (2019) Quercetin improves nonalcoholic fatty liver by ameliorating inflammation, oxidative stress, and lipid metabolism in db/db mice. Phytother Res 33(12): 3140–3152
98. Lu K, Qiao R et al (2018) Influence of microplastics on the accumulation and chronic toxic effects of cadmium in zebrafish (Danio rerio). Chemosphere 202:514–520
99. Lebreton LC, van Der Zwet J et al (2017) River plastic emissions to the world's oceans. Nat Commun 8(1):1–10
100. Cole M, Lindeque P et al (2013) Microplastic ingestion by zooplankton. Environ Sci Technol 47(12):6646–6655
101. Watts AJ, Lewis C et al (2014) Uptake and retention of microplastics by the shore crab Carcinus maenas. Environ Sci Technol 48(15):8823–8830

102. Wright SL, Thompson RC, Galloway TS (2013) The physical impacts of microplastics on marine organisms: a review. Environ Pollut 178:483–492
103. Awuchi CG, Awuchi CG (2019) Impacts of plastic pollution on the sustainability of seafood value chain and human health. Int J Adv Acad Res 5(11):46–138
104. Alimba CG, Faggio C (2019) Microplastics in the marine environment: current trends in environmental pollution and mechanisms of toxicological profile. Environ Toxicol Pharmacol 68:61–74
105. Prokin AA, Dubov PG, Bolotov SE (2015) Formation of macroinvertebrates communities in duckweed (Lemnaceae) and artificial surface-floating substrate: results of the experiment under natural conditions. Inland Water Biol 8(4):373–383
106. Besseling E, Wang B et al (2014) Nanoplastic affects growth of S. obliquus and reproduction of D. magna. Environ Sci Technol 48(20):12336–12343
107. Dovidat LC, Brinkmann BW et al (2020) Plastic particles adsorb to the roots of freshwater vascular plant Spirodela polyrhiza but do not impair growth. Limnol Oceanograph Lett 5(1): 37–45
108. Cunha C, Paulo J et al (2019) Ecotoxicological and biochemical effects of environmental concentrations of the plastic-bond pollutant dibutyl phthalate on Scenedesmus sp. Aqua Toxicol 215:105281
109. Zhang C, Chen X et al (2017) Toxic effects of microplastic on marine microalgae Skeletonema costatum: interactions between microplastic and algae. Environ Pollut 220L:1282–1288
110. Kalčíková G, Skalar T et al (2020) An environmental concentration of aged microplastics with adsorbed silver significantly affects aquatic organisms. Water Res 175:115644
111. Xia X, Sun M et al (2020) Polyvinyl chloride microplastics induce growth inhibition and oxidative stress in Cyprinus carpio var. larvae. Sci Total Environ 716:136479
112. Guimarães ATB, Charlie-Silva I, Malafaia G (2021) Toxic effects of naturally-aged microplastics on zebrafish juveniles: a more realistic approach to plastic pollution in freshwater ecosystems. J Hazard Mater 407:124833
113. Cormier B, Gambardella C et al (2021) Chemicals sorbed to environmental microplastics are toxic to early life stages of aquatic organisms. Ecotoxicol Environ Safe 208:111665
114. Yu H, Zhang X et al (2020) Ecotoxicity of polystyrene microplastics to submerged carnivorous Utricularia vulgaris plants in freshwater ecosystems. Environ Pollut 265:114830
115. Trestrail C, Walpitagama M et al (2020) Foaming at the mouth: ingestion of floral foam microplastics by aquatic animals. Sci Total Environ 705:135826
116. Shiu RF, Vazquez CI et al (2020) Nano-and microplastics trigger secretion of protein-rich extracellular polymeric substances from phytoplankton. Sci Total Environ 748:141469
117. Long M, Moriceau B et al (2015) Interactions between microplastics and phytoplankton aggregates: impact on their respective fates. Mar Chem 175:39–46
118. Long M, Paul-Pont I et al (2017) Interactions between polystyrene microplastics and marine phytoplankton lead to species-specific hetero-aggregation. Environ Pollut 228:454–463
119. Sjollema SB, Redondo-Hasselerharm P et al (2016) Do plastic particles affect microalgal photosynthesis and growth? Aqua Toxicol 170:259–261
120. Enders K, Lenz R et al (2015) Abundance, size and polymer composition of marine microplastics≥ 10 μm in the Atlantic Ocean and their modelled vertical distribution. Mar Pollut Bull 100(1):70–81
121. Lee H, Shim WJ, Kwon JH (2014) Sorption capacity of plastic debris for hydrophobic organic chemicals. Sci Total Environ 470:1545–1552
122. Revel M, Châtel A, Mouneyrac C (2018) Micro (nano) plastics: a threat to human health? Curr Opin Environ Sci Health 1:17–23
123. Cox KD, Covernton GA et al (2019) Human consumption of microplastics. Environ Sci Technol 53(12):7068–7074
124. PlasticsEurope, 2019. Plastics—the facts 2019. An analysis of European plastics production, demand and waste data. https://www.plasticseurope.org/application/files/1115/7236/4388/FINAL_web_version_Plastics_the_facts2019_14102019.pdf (accessed May 01, 2020)

125. Allen S, Allen D et al (2019) Atmospheric transport and deposition of microplastics in a remote mountain catchment. Nat Geosci 12(5):339–344
126. Prata JC (2018) Airborne microplastics: consequences to human health? Environ Pollut 234: 115–126
127. Wright SL, Kelly FJ (2017) Plastic and human health: a micro issue? Environ Sci Technol 51(12):6634–6647
128. Law BD, Bunn WB, Hesterberg TW (1990) Solubility of polymeric organic fibers and manmade vitreous fibers in gambles solution. Inhal Toxicol 2(4):321–339
129. Washko RM, Day B et al (2000) Epidemiologic investigation of respiratory morbidity at a nylon flock plant. Am J Ind Med 38(6):628–638
130. Valavanidis A, Vlachogianni T et al (2013) Pulmonary oxidative stress, inflammation and cancer: respirable particulate matter, fibrous dusts and ozone as major causes of lung carcinogenesis through reactive oxygen species mechanisms. Int J Environ Res Public Health 10(9): 3886–3907
131. Cho YJ, Park SB et al (2018) Bisphenol A modulates inflammation and proliferation pathway in human endometrial stromal cells by inducing oxidative stress. Reprod Toxicol 81:41–49
132. Pérez-Albaladejo E, Solé M and Porte C (2020) Plastics and plastic additives as inducers of oxidative stress. Curr Opin Toxicol
133. Abbas-Abadi MS, Jalali A et al (2020) The atmospheric, vacuum and pressurized pyrolysis of used bleaching soils along with polymeric wastes to reach the valuable and economical fuels. J Clean Prod 255:120328
134. Chandra, Rustgi R (1998) Biodegradable polymers. Progress Polym Sci 23(7):1273–1335
135. Anunciado MB, Hayes DG et al (2021) Effect of environmental weathering on biodegradation of biodegradable plastic mulch films under ambient soil and composting conditions. J Polym Environ:1–16
136. Rujnić-Sokele M, Pilipović A (2017) Challenges and opportunities of biodegradable plastics: a mini review. Waste Manag Res 35(2):132–140
137. Tokiwa Y, Calabia BP et al (2009) Biodegradability of plastics. Int J Mol Sci 10(9):3722–3742
138. Park SY, Kim CG (2019) Biodegradation of micro-polyethylene particles by bacterial colonization of a mixed microbial consortium isolated from a landfill site. Chemosphere 222:527–533
139. Auta HS, Emenike CU, Fauziah SH (2017) Screening of Bacillus strains isolated from mangrove ecosystems in Peninsular Malaysia for microplastic degradation. Environ Pollut 231:1552–1559
140. Auta HS, Emenike CU et al (2018) Growth kinetics and biodeterioration of polypropylene microplastics by Bacillus sp. and Rhodococcus sp. isolated from mangrove sediment. Mar Pollut Bull 127:15–21
141. Janssen PH, Yates PS et al (2002) Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. Appl Environ Microbiol 68(5):2391–2396
142. Yuan J, Ma J et al (2020) Microbial degradation and other environmental aspects of microplastics/plastics. Sci Total Environ 715:136968
143. Pathak VM, Kumar N (2017) Implications of SiO 2 nanoparticles for in vitro biodegradation of low-density polyethylene with potential isolates of bacillus, pseudomonas, and their synergistic effect on Vigna mungo growth. Energy Ecol Environ 2(6):418–427
144. Russell JR, Huang J et al (2011) Biodegradation of polyester polyurethane by endophytic fungi. Appl Environ Microbiol 77(17):6076–6084
145. Yamada-Onodera K, Mukumoto H et al (2001) Degradation of polyethylene by a fungus, *Penicillium simplicissimum*. YK Polym Degrad Stabil 72(2):323–327
146. Bhardwaj H, Gupta R, Tiwari A (2013) Communities of microbial enzymes associated with biodegradation of plastics. J Polym Environ 21(2):575–579

147. Zafar U, Houlden A, Robson GD (2013) Fungal communities associated with the biodegradation of polyester polyurethane buried under compost at different temperatures. Appl Environ Microbiol 79(23):7313–7324
148. Lee SY, Ten LN et al (2021) Biodegradative activities of fungal strains isolated from terrestrial environments in Korea. Mycobiology:1–9
149. Oviedo-Anchundia R, del Castillo DS et al (2021) Analysis of the degradation of polyethylene, polystyrene and polyurethane mediated by three filamentous fungi isolated from the Antarctica. African J Biotechnol 20(2):66–76
150. DSouza GC, Sheriff RS et al (2021) Fungal biodegradation of low-density polyethylene using consortium of Aspergillus species under controlled conditions. Heliyon 7(5):e07008
151. Chaudhary AK, Vijayakumar RP (2020) Effect of chemical treatment on biological degradation of high-density polyethylene (HDPE). Environ Dev Sustain 22(2):1093–1104
152. da Luz JMR, da Silva MDCS et al. (2019) Plastics polymers degradation by fungi. In Microorganisms. IntechOpen
153. Magnin A, Hoornaert L et al (2019) Isolation and characterization of different promising fungi for biological waste management of polyurethanes. Microbial Biotechnol 12(3):544–555
154. Brunner I, Fischer M et al (2018) Ability of fungi isolated from plastic debris floating in the shoreline of a lake to degrade plastics. PLoS One 13(8):e0202047
155. Munir E, Harefa RSM et al (2018) Plastic degrading fungi Trichoderma viride and aspergillus nomius isolated from local landfill soil in Medan. In IOP conference series: earth and environmental science. IOP Publishing 126(1):012145
156. Khan S, Nadir S et al (2017) Biodegradation of polyester polyurethane by aspergillus tubingensis. Environ Pollut 225:469–480
157. Ojha N, Pradhan N et al (2017) Evaluation of HDPE and LDPE degradation by fungus, implemented by statistical optimization. Sci Rep 7(1):1–13
158. Alshehrei F (2017) Biodegradation of low density polyethylene by fungi isolated from Red Sea water. Int J Curr Microbiol App Sci 6(8):1703–1709
159. Álvarez-Barragán J, Domínguez-Malfavón L et al (2016) Biodegradative activities of selected environmental fungi on a polyester polyurethane varnish and polyether polyurethane foams. Appl Environ Microbiol 82(17):5225–5235
160. Devi RS, Kannan VR et al (2015) Biodegradation of HDPE by Aspergillus spp. from marine ecosystem of Gulf of Mannar, India. Mar Pollut Bull 96(1–2):32–40
161. Krueger MC, Hofmann U et al (2015) Potential of wood-rotting fungi to attack polystyrene sulfonate and its depolymerisation by *Gloeophyllum trabeum* via hydroquinone-driven Fenton chemistry. PLoS One 10(7):e0131773
162. Sheik S, Chandrashekar KR et al (2015) Biodegradation of gamma irradiated low density polyethylene and polypropylene by endophytic fungi. Int Biodeter Biodegrad 105:21–29
163. Sowmya HV, Krishnappa M, Thippeswamy B (2015) Degradation of polyethylene by *Penicillium simplicissimum* isolated from local dumpsite of Shivamogga district. Environ Dev Sustain 17(4):731–745
164. Balasubramanian V, Natarajan K et al (2014) Enhancement of in vitro high-density polyethylene (HDPE) degradation by physical, chemical, and biological treatments. Environ Sci Pollut Res 21(21):12549–12562
165. Tachibana K, Hashimoto K et al (2010) Isolation and characterization of microorganisms degrading nylon 4 in the composted soil. Polym Degrad Stab 95(6):912–917
166. Ronkvist ÅM, Xie W et al (2009) Cutinase-catalyzed hydrolysis of poly (ethylene terephthalate). Macromolecules 42(14):5128–5138
167. Friedrich J, Zalar P et al (2007) Ability of fungi to degrade synthetic polymer nylon-6. Chemosphere 67(10):2089–2095
168. Nimchua T, Punnapayak H, Zimmermann W (2007) Comparison of the hydrolysis of polyethylene terephthalate fibers by a hydrolase from Fusarium oxysporum LCH I and Fusarium solani f. sp pisi. Biotechnol J Healthcare Nutrit Technol 2(3):361–364

169. Iiyoshi Y, Tsutsumi Y, Nishida T (1998) Polyethylene degradation by lignin-degrading fungi and manganese peroxidase. J Wood Sci 44(3):222–229
170. Deguchi T, Kakezawa M, Nishida T (1997) Nylon biodegradation by lignin-degrading fungi. Appl Environ Microbiol 63(1):329–331
171. Zhang J, Gao D et al (2020) Biodegradation of polyethylene microplastic particles by the fungus aspergillus flavus from the guts of wax moth *Galleria mellonella*. Sci Total Environ 704:135931
172. Spina F, Tummino ML et al (2021) Low density polyethylene degradation by filamentous fungi. Environ Pollut 274:116548
173. Castiglia VC, Kuhar F (2015) Deterioration of expanded polystyrene caused by *Aureobasidium pullulans* var. *melanogenum*. Rev Argent Microbiol 47(3):256–260
174. Yanto DHY, Krishanti NPRA et al (2019) Biodegradation of styrofoam waste by ligninolytic fungi and bacteria. In IOP conference series: earth and environmental science. IOP Publishing 308(1):012001
175. Othman AR, Hasan HA et al (2021) Microbial degradation of microplastics by enzymatic processes: a review. Environ Chem Lett 19(4):3057–3073
176. Ali MI, Ahmed S et al (2014) Biodegradation of starch blended polyvinyl chloride films by isolated Phanerochaete chrysosporium PV1. Int J Environ Sci Technol 11(2):339–348
177. Sumathi T, Viswanath B et al (2016) Production of laccase by *Cochliobolus* sp. isolated from plastic dumped soils and their ability to degrade low molecular weight PVC. Biochem Res Int
178. Mathur G, Prasad R (2012) Degradation of polyurethane by Aspergillus flavus (ITCC 6051) isolated from soil. Appl Biochem Biotechnol 167(6):1595–1602
179. Cosgrove L, McGeechan PL et al (2007) Fungal communities associated with degradation of polyester polyurethane in soil. Appl Environ Microbiol 73(18):5817–5824
180. Osman M, Satti SM et al (2018) Degradation of polyester polyurethane by aspergillus sp. strain S45 isolated from soil. J Polym Environ 26(1):301–310
181. Chen WH, Vázquez-González M et al (2018) Biocatalytic cascades driven by enzymes encapsulated in metal–organic framework nanoparticles. Nat Catal 1(9):689–695
182. Austin HP, Allen MD et al (2018) Characterization and engineering of a plastic-degrading aromatic polyesterase. Proc Natl Acad Sci 115(19):E4350–E4357
183. Delamarche E, Mattlet A et al (2020) Tailoring biodegradability of poly (butylene succinate)/poly (lactic acid) blends with a deep eutectic solvent. Front Mater 7:7
184. Rodríguez-Seijo A, da Costa JP et al (2018) Oxidative stress, energy metabolism and molecular responses of earthworms (Eisenia fetida) exposed to low-density polyethylene microplastics. Environ Sci Pollut Res 25(33):33599–33610
185. McKeown P, Jones MD (2020) The chemical recycling of PLA: a review. Sustain Chem 1:1–22
186. Crabbe JR, Campbell JR et al (1994) Biodegradation of a colloidal ester-based polyurethane by soil fungi. Int Biodeter Biodegrad 33(2):103–113
187. Nakajima-Kambe T, Shigeno-Akutsu Y (1999) Microbial degradation of polyurethane, polyester polyurethanes and polyether polyurethanes. Appl Microbiol Biotechnol 51(2):134–140
188. Mohanan N, Montazer Z et al (2020) Microbial and enzymatic degradation of synthetic plastics. Front Microbiol 11:2837

Part II

Fungal Metabolic Products

A Bird's-Eye View of Fungal Peptides

6

V. Sabareesh and V. S. Gowri

Abstract

Peptide-based drugs/antibiotics are increasingly becoming preferable due to greater incidence of multidrug resistance towards non-peptidic drugs. In addition, several non-peptidic compounds also elicit unfavorable or toxic side effects. Consequently, besides bacteria, plants and animal-based sources, fungi have also been explored very much in the search for novel therapeutic peptides that can be more efficacious and safer. Based on the biosynthetic pathways, these peptides can be classified as (i) ribosomal peptides and (ii) non-ribosomal peptides (NRPs). Several NRPs from diverse fungal sources (soil, marine, and endophytes) have been identified and comprehensively characterized, whereas studies on ribosomal peptides of fungal origin are limited. In terms of molecular architecture, fungal peptides can be classified into four major categories: (i) linear peptides with modified N- and C-termini (e.g., peptaibiotics and peptaibols); (ii) head-to-tail (backbone) cyclized peptides; (iii) cyclic depsipeptides; and (iv) sidechain cyclized peptides. In this chapter, salient features of some major fungal peptides that have been found to elicit various kinds of biological activities are presented. A few aspects on some fungal peptide-based products of pharmaceutical or medical relevance, e.g., caspofungin (brand name: Cancidas) and cyclosporin A, which are already available in the market, are also briefly delineated.

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Fungi · Drugs · Antimicrobial peptides · Linear peptides · Cyclic peptides · Defensins

6.1 Introduction

Right from the time of serendipitous finding of penicillin till now, fungi have always been sought after for discovering several drug compounds. Fungi also have been useful as biocontrol agents, particularly for agricultural applications [1]. Among various types of bioactive molecules that have been identified from different fungal species, peptides constitute an important class. Fungal peptides not only elicit very interesting in vitro biological functions, but some have also been sold as drugs, e.g., caspofungin (brand name: Cancidas; Drug Bank Accession No. DB00520) and micafungin (brand name: Mycamine, Drug Bank Accession No. DB01141), while some have been found to be useful in the preclinical stages of drug discovery, e.g., NZ2114, a derivative of a defensin plectasin [2]. The fungal peptides encompass both ribosomal and non-ribosomal classes, depending on the biosynthetic pathways. The peptides synthesized by the conventional ribosomal machinery are called as RiPPs, which means "Ribosomally synthesized and posttranslationally modified peptides" [3–5]. When compared to the non-ribosomal peptides from fungi, only a few studies on fungal RiPPs are available in the literature, and those are relatively recent [5]. Amatoxins and borosins are some examples of fungal RiPPs. The biosynthesis of non-ribosomal peptides involves very large multi-modular enzymes, called non-ribosomal peptide synthetases (NRPSs) [6, 7]. The NRPSs utilize both proteinogenic and non-proteinogenic amino acids in the biosynthesis of non-ribosomal peptides (NRPs). α-Amino isobutyric acid (Aib), isovaline (Iva), γ-amino butyric acid (GABA), and anthranilic acid (ATA) are a few examples of non-proteinogenic amino acids that are found in several NRPs. Additionally, several fungal NRPSs also engage "hydroxy acids" during biosynthesis, thereby producing a different class of NRPs called "depsipeptides" [8]. Because of the incorporation of the hydroxy acid, depsipeptides contain at least "an ester bond" in their backbone, in addition to the usual presence of the peptide/amide bonds.

Based on the molecular structure/architecture, fungal peptides can be classified into four major categories: (i) linear peptides with modified N- and C-termini; (ii) head-to-tail (backbone) cyclized peptides; (iii) cyclic depsipeptides; and (iv) sidechain cyclized peptides. Cyclic depsipeptides would encompass both backbone and sidechain cyclized peptides. The category, sidechain cyclized peptides, mostly pertains to disulfide-bonded peptides. The differences in the molecular architecture between these four categories can be understood from Fig. 6.1.

Fungal peptides have been identified from diverse species that inhabit various ecosystems such as soil, marine, and plants (endophytes) and also those that thrive in insect hosts, e.g., entomopathogens [9, 10]. Since several fungal NRPs have modified/blocked N- and C-termini including the cyclic peptides, sequence

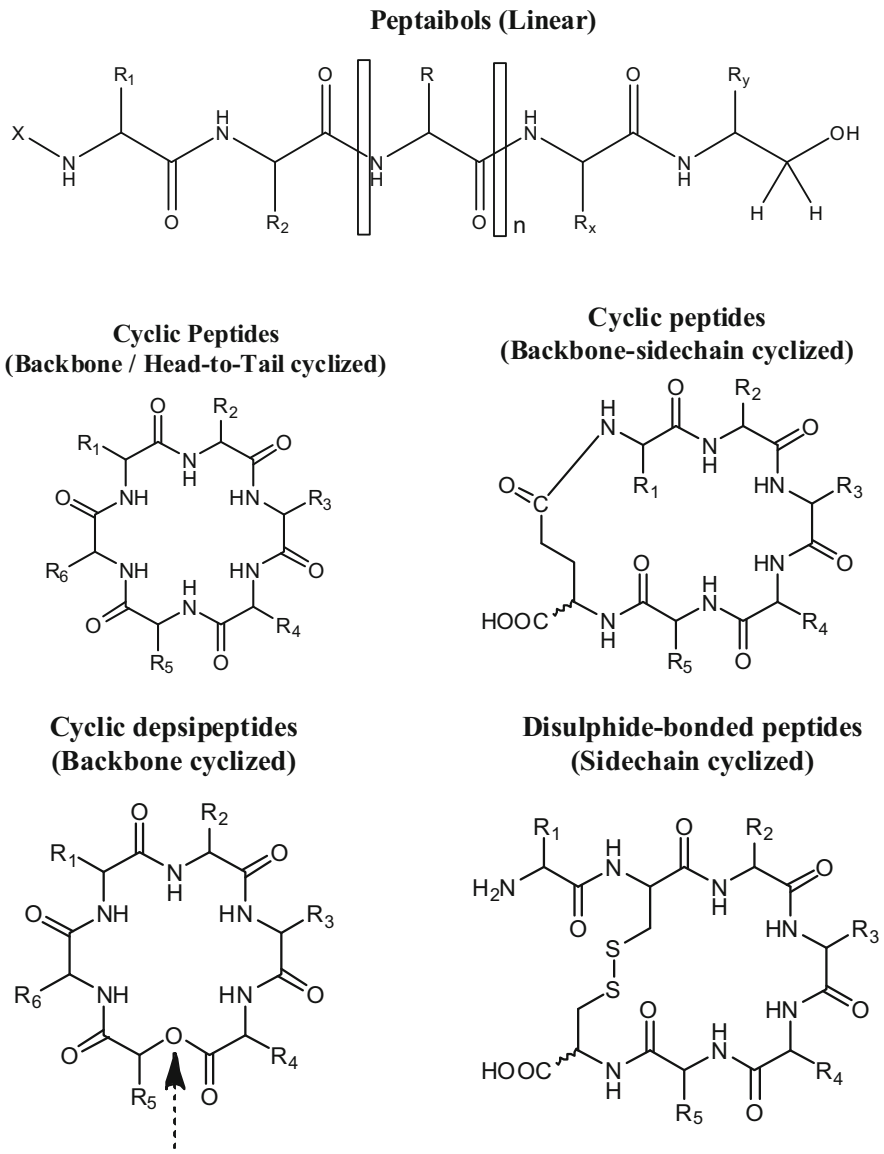

Fig. 6.1 Molecular architectures of fungal peptides

elucidation of such peptides has been usually carried out by spectroscopic methods rather than by Edman's degradation method (N-terminal sequencing). Nuclear magnetic resonance (NMR) spectroscopy involving homo- and hetero-nuclear multidimensional methods and mass spectrometry (MS)-based methods have proven to be successful in deducing the molecular structure and the sequence of several fungal NRPs. Marfey's method has been widely applied for determining stereochemical

configuration of the constituting amino acid residues. High-performance liquid chromatography (HPLC), particularly reverse phase (RP)-HPLC, has been instrumental in advancing as well as accelerating the process of discovering many NRPs of fungal origin, especially to isolate pure peptidic components for the purpose of carrying out biological assays. Further, combining chromatography with MS too has been immensely fruitful not only for sequencing various fungal NRPs but also to identify several variants, which possess microheterogeneous sequences. In the earlier years, gas chromatography (GC)-MS was utilized for sequencing [11–14], and of late, especially during the last three decades or so, RP-HPLC and MS have been increasingly applied for identifying and sequencing fungal NRPs [15–17]. Online LC-MS also has been attempted to identify and characterize fungal peptides [18–22]. Salient features including molecular structural properties and biological function of both non-ribosomal and ribosomal fungal peptides are presented in this chapter.

6.2 Non-ribosomal Fungal Peptides

6.2.1 Peptaibiotics: Peptaibols (Linear NRPs)

Many fungi produce linear peptides having a very high content of Aib (c.f. vide supra) called "peptaibiotics" [23]. A major class within peptaibiotics is constituted by "peptaibols," which possess acetylated N-termini and C-terminus alcohol [24]. The name "peptaibol" was proposed/coined considering the prevalence of "Aib" in the sequences and the presence of the C-terminus alcohol group in these peptides, viz., "pept + aib + ol" [25]. In addition to Aib, several peptaibols are also known to have "isovaline" (ethylalanine), which is another α,α-dialkyl α-amino acid similar to Aib [26, 27]. Thus far, more than 700 peptaibols have been identified, and their lengths are in the range 4–22 including the C-terminus alcohol residue [22, 28–32].

With regard to the source, relatively a larger number of peptaibols have been identified from *Trichoderma* species [22, 25, 30, 32]. In addition to the soil fungi, peptaibols have been discovered from marine fungi as well, e.g., short-length trichobrachins A and B containing 11 residues and long-chain peptaibols composed of 20 residues have been reported from marine-derived strain of *Trichoderma longibrachiatum* [33, 34]. Recently, five new 15-residue-long pentadecaibins I–V were reported from marine-derived *Trichoderma* sp. of the *Harzianum* clade [35]. Further, various peptaibols have been reported from *Emericellopsis* sp., *Cephalosporium pimprina* Thirum., and *Acremonium* sp. also [12–14].

Peptaibols have the ability to insert into the lipid bilayer architecture of the cellular membranes and form transmembrane ion channels or pores [36, 37]. So, peptaibols are also called as "channel-forming peptides (CFPs)," which can serve as models to understand the behavior of ion-channeling activity that is usually carried out by transmembrane (receptor) proteins [36, 38]. Most of the channels formed by the peptaibols get activated upon application of electric field, and hence, they are

also referred to as voltage-dependent ion channels [38]. The first peptaibol that was subjected to voltage-dependent studies was alamethicin [39]. Incidentally, alamethicin is also the first peptaibol, whose three-dimensional (3D) molecular structure was elucidated by X-ray diffraction, which was reported by Fox and Richards in 1982 (vide infra) [40]. This 3D molecular structure could provide insights into mechanisms of ion-channel formation aiding in the translocation of metal ions across the membrane. As a representative example, the molecular structure of 20-residue-long alamethicin F-30 is shown in Fig. 6.2a. Alamethicin F-30 has eight Aib residues and C-terminus phenylalaninol. 3D molecular structures of several other natural peptaibols and synthetic analogues were also determined by X-ray diffraction, NMR spectroscopy, and circular dichroism spectroscopy [41–50].

Most of these structures have been found to adopt largely helical conformations [51, 52]. Though Aib is known to predominantly nucleate helical conformations, it gets accommodated in other types of conformations, e.g., polyproline II and extended conformations also, as reviewed by Aravinda et al. (2008) [51].

In terms of primary structure, peptaibols are often produced as mixtures of very closely related peptides that have microheterogeneous sequences (i.e., isoforms), which may be attributed to their biosynthetic route that involve NRPSs [27]. The microheterogeneity can be due to exchanges or replacements of amino acids such as Gly ↔ Ala, Ala ↔ Aib, Aib ↔ D-Iva, and Gln ↔ Glu, only at certain specific positions in their sequences, which causes differences of 1 Dalton (Da) or 14 Da or 15 Da between their molecular masses. Often such replacements do not even alter their molecular masses, but only lead to some minor variations in their sequences, viz., isobaric analogues. Another striking observation is that the occurrence of the dipeptide fragments Aib-Aib and Aib-Pro is preponderant in several peptaibol sequences [53]. Based on amino acid sequence identities, peptaibols are classified into nine superfamilies. Position-specific preferences of amino acids and the lengths of the peptides distinguish the members between the superfamilies [52].

Lipopeptaibols and Other Peptaibiotics Other than acetylated N-terminus, peptides possessing N-terminus acyl modification, e.g., octanoyl, dodecanoyl, etc., also have been identified from some fungi. These are called "lipopeptaibols," e.g., trichogin A IV from *Trichoderma longibrachiatum* [41], trikoningins KB I and II from *Trichoderma koningii* Oudem. (collected in Uruguay) [54], halovirs from the marine fungus of the genus *Scytalidium* [55], and lipovelutibols from the Himalayan *Trichoderma velutinum* [17]. Further, instead of C-terminus alcohol, fungal peptaibiotics with C-terminus modified by amine or amide functional group too have been discovered, for instance, leucinostatins, efrapeptins (Fig. 6.2b) and trichopolyns [26, 56]. The C-termini of efrapeptins contain an unusual moiety: 1,5-diazabicyclo [4:3:0] nonene (DBN) [57–59] (Fig. 6.2b). Presence of "pipecolic acid" and "β-alanine" residues are some notable features in the sequences of efrapeptins, leucinostatins, and gichigamins [26, 28, 57, 58]. Fig. 6.2b shows a representative molecular structure of an efrapeptin.

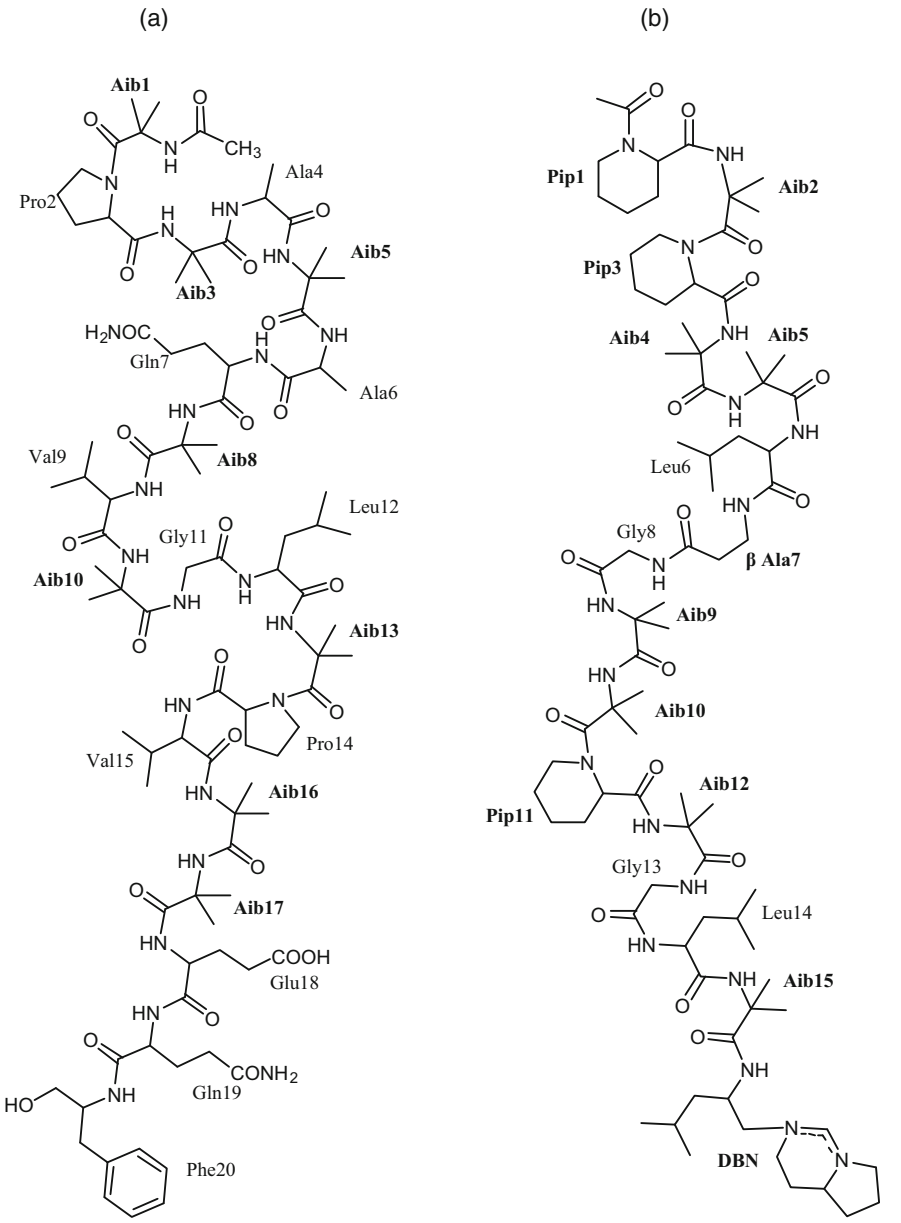

Fig. 6.2 Molecular structures of (**a**) alamethicin F-30 (peptaibol) and (**b**) efrapeptin C (peptaibiotic). In addition to Aib, presence of pipecolic acid (Pip), β-Ala, and C-terminus DBN can be noted in efrapeptin C

Sequence Elucidation of Peptaibiotics In the 1970s, Rinehart's group began to elucidate the sequences of alamethicins, emerimicins, and antiamoebin by using gas chromatography-mass spectrometry (GC-MS), involving field desorption (FD) as the mode of ionization [12–14]. Subsequently, in the 1980s, fast atom bombardment (FAB)-MS was utilized to deduce the sequences of other peptaibols, e.g., zervamicins, trichotoxins, paracelsins, etc. [60, 61]. HPLC, particularly the reverse phase mode in conjunction with FD and FAB-MS, also was shown to be useful to identify various classes of microheterogeneous peptaibols [11]. From the late 1990s, electrospray ionization (ESI)-MS began to be applied for identifying novel peptaibol molecules and also to characterize the extent of microheterogeneity in their sequences [15, 18, 19, 21, 33, 59, 62, 63]. NMR spectroscopic methods are also applied to verify the sequences as well as to determine the 3D conformation [16, 30, 32, 35]. The stereochemical configurations of the constituting amino acids are determined by using Marfey's method [16, 17, 30, 32, 35].

Biological and Other Functions

As aforementioned (vide supra), several peptaibols can form ion channels by inserting into the cellular membranes and aid in the translocation of cations across the membrane [36, 38]. Based on the size of the peptaibols, different models were put forward in order to understand the mechanism of formation of ion channels within the cell membrane. "Barrel stave model" has been suggested for longer peptaibols (~17–20 amino acid residues) such as alamethicin, whereas "Carpet model" was proposed for short peptaibol sequences, e.g., trichogin A IV [52]. Further, peptaibols have also been demonstrated to be "uncouplers" of mitochondrial oxidative phosphorylation [64, 65]. Besides their insect toxicity, the efrapeptins (peptaibiotics) from the entomopathogenic *Tolypocladium niveum* strongly inhibit mitochondrial (F1) ATPase and photophosphorylation in chloroplasts [57, 66]. The peptaibols, e.g., trichocellins A-II and B-II, have been shown to induce catecholamine secretion from bovine adrenal chromaffin cells through Ca^{2+} influx, in addition to their ability to form voltage-dependent ion channels in bilayer lipid membranes [67]. Interestingly, a peptaibol from a *Trichoderma* sp. has been found to inhibit the formation of amyloid β-peptide in cultured neuron cells of primary guinea pig cerebral cortex, at sub-μM concentrations ($IC_{50} = 0.1$ μg/ml), and it was not observed to be cytotoxic as well for concentrations <3 μg/ml [68].

Peptaibols possessing antiviral activity too have been reported. Rowley et al. discovered a few lipopeptaibols called "halovirs," which were potent inhibitors of herpex simplex viruses (HSV) 1 and 2 in vitro, at low and sub-micromolar concentrations [55]. The halovirs were found to be more active than the free fatty acids, thereby showing the importance of the hexapeptide portion for the potent virucidal activity. Studies on peptaibols' activity against mammalian cells are relatively less. In this regard, a 22-mer peptaibol, gichigamin A, and some of its related analogs were investigated on a pancreatic cancer cell line (MIA PaCa-2) in vitro, and it was observed that these peptaibols exhibited a wide range of antiproliferative and cytotoxic potencies [28]. Motivated from the outcomes of

these in vitro experiments, gichigamin A was chosen for in vivo experiments, which showed that this peptaibol had significant and potent anti-tumor activity in a MIA PaCa-2 xenograft mouse model. Moderate in vitro cytotoxic activities against a panel of human cancer cell lines (HL-60, LS180, MDA-MB-231, and A549) were observed with two lipovelutibols that were isolated from the Himalayan cold habitat fungus *Trichoderma velutinum* [17]. Further, a 14-residue peptaibol from the same Himalayan psychrotrophic *Trichoderma velutinum* showed cytotoxic activity against the cancer cell lines, HL-60 and MDA-MB-231, with IC_{50} values 4 and 7 μM, respectively [16]. In the HL-60 cells, the same 14-residue peptaibol showed apoptosis in a dose-dependent manner [16]. Recently, a few peptaibols identified from a sponge-derived *Acremonium* sp. have also been observed to exhibit cytotoxic activity against A549 and/or HepG2 cancer cell lines [29]. Additionally, efrapeptin J (peptaibiotic) from a marine *Tolypocladium* sp. has been shown to be a downregulator of a molecular chaperone GRP78, which is implicated in anti-tumor activity, whereby these experiments were done in HT1080 cells and in MKN-74 human gastric cancer cells [69].

Interestingly, peptaibols have been shown to be useful in the microbial fuel cells (MFC) also. Because of their antibacterial activity, peptaibols (neoatroviridins A–D from *Trichoderma viride*) have been shown to aid in improving the power generation of MFCs by inhibiting methanogenic bacteria [70].

6.2.2 Cyclic Peptides (NRPs)

About 290 fungal cyclic peptides have been reported in the literature, which has been reviewed by Wang et al. (2017) [9], wherein they have classified these cyclic peptides according to their size, viz., based on the number of amino acid residues constituting the cyclic peptide. Starting from cyclic tripeptides (composed of three residues), Wang et al. (2017) [9] have covered cyclic peptides of sizes up to 18 amino acid residues. Many are head-to-tail (viz., fully backbone) cyclized peptides; however, considerable number of "backbone-sidechain cyclized peptides" have also been identified. In the ensuing discussions, salient features of certain classes of cyclic peptides, especially those possessing some rare amino acid residues, are described.

Cyclic peptides have been found from the soil fungi, marine fungi [10], entomopathogenic fungi, as well as endophytic fungi [9]. To a larger extent, fungal cyclic peptides have been identified from the genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Acremonium* [9]. Cyclic peptides have been reported from psychrophilic and psychrotolerant fungi as well. In this regard, a very interesting cyclic peptide called "psychrophilin A" is of worth to be mentioned, which was identified from psychrotolerant *Penicillium ribeum* [71]. Psychrophilin A is the first natural cyclic peptide reported to contain a nitro group (instead of amino group). Another notable feature of psychrophilin A is, the presence of anthranilic acid (ATA), whose amino and carboxylic acid groups are part of the cyclic backbone structure, whereby the carboxylic group of the anthranilic acid forms a peptide bond with the nitrogen of

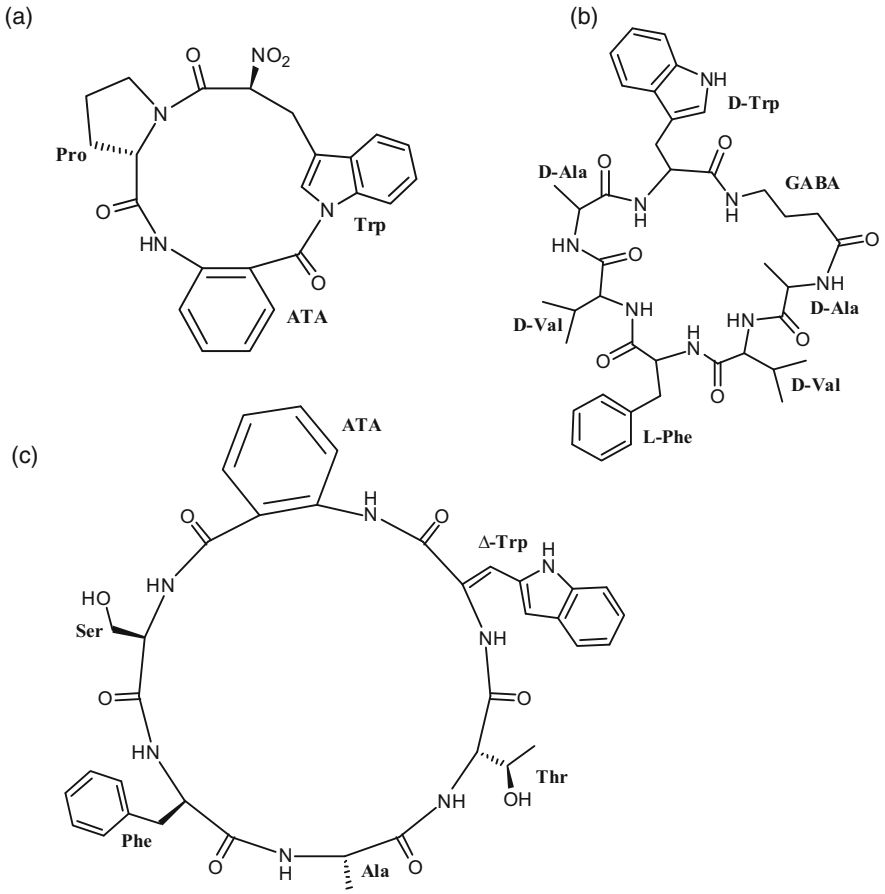

Fig. 6.3 Structure of cyclic peptides. (**a**) Psychrophillin A. (**b**) Unguisin A. (**c**) Sclerotide A. *ATA* anthranilic acid, *GABA* gamma aminobutyric acid, *Δ-Trp* dehydrotryptophan

the tryptophan sidechain (see Fig. 6.3a). Thus, instead of an amino group, a nitro group is actually linked to the α-carbon of the tryptophan residue. The amino group of the anthranilic acid forms a peptide bond with carboxyl group of proline. Fig. 6.3a shows this unusual molecular structure of psychrophilin A. Two more cyclic peptides, cycloaspeptides A and D, also were identified from the same psychrotolerant *Penicillium ribeum* [71]. These two peptides also contain ATA as part of the cyclic backbone structure.

Additionally, the cycloaspeptides A and D possess N-methylated residues: N-methyl phenylalanine and N-methyl tyrosine. Another psychrotolerant species, *Penicillium algidum*, was found to produce "psychrophilin D" [72], which has leucine residue instead of proline of psychrophilin A. The psychrophilin D was found to exhibit moderate activity in the P388 murine leukemia cell assay, while the cycloaspeptides A and D showed moderate activity against *Plasmodium falciparum*

[72]. Further, four new cyclic peptides that were very closely related to psychrophilin class of peptides (as discussed above) were discovered from marine-derived fungus *Aspergillus versicolor* ZLN-60 [73]. From this same species of marine *Aspergillus*, Peng et al. (2014) also identified a hexapeptide, versicotide C that had two ATA residues and two N-methylated alanine residues. Two new cyclic peptides containing γ-Aminobutyric acid (GABA), unguisins A (Fig. 6.3b) and B, were identified from the marine-derived *Emericella unguis* by Malmstrom (1999), and this was the first study that reported about the occurrence of GABA as part of the ring structure of the cyclic heptapeptides [74]. An intriguing aspect is that five residues of these cycloheptapeptides have D-stereochemical configuration.

Subsequently, unguisins C and D were also reported from the same marine *Emericella* sp., whereby the peptide unguisin D was obtained through precursor-directed biosynthesis by adding L-leucine in the fermentation medium, as a result of which leucine got incorporated in the sequence of unguisin D, by replacing one of the valines of unguisin B [75]. Further, unguisin F was reported from an endophytic fungus *Mucor irregularis*, which was isolated from a medicinal plant *Moringa stenopetala* [76]. Aib containing cyclic heptapeptides, scytalidamides A and B, have also been reported from a marine fungus *Scytalidium*, wherein the scytalidamide B possessed 3-methyl proline residue, which is a rare modification [77]. Both these peptides showed in vitro cytotoxicity towards human colon carcinoma tumor cell line HCT-116 and NCI-60 cell line panel, with the IC_{50} values in the range 2–11 μM [77].

Sclerotides A and B from a marine-derived halotolerant *Aspergillus sclerotiorum* PT06-1 are interesting examples of cyclic hexapeptides in that they possess an unusual amino acid, dehydrotryptophan (Δ-Trp) [78]. Sclerotides A and B also contain ATA (Fig. 6.3c). Another notable aspect is that the sclerotide A and sclerotide B were photointerconvertible, whereby this inter-conversion occurs at the double bond of the Δ-Trp. And recently, three novel sclerotides C–E have been reported from soft coral-derived *Aspergillus sclerotiorum* SCSIO 41031 [79]. The same marine halotolerant *Aspergillus sclerotiorum* PT06-1 produced another class of cyclic peptides, which contained "ornithine" and N-methylated residues, in a nutrient-rich medium [80]. These were cyclic tripeptides named as "sclerotiotides," wherein the δ-amino group of ornithine was involved in the formation of backbone amide (peptide) bond with the next amino acid residue. However, the α-amino group of the ornithine residue is not free; rather, it is bonded to a fatty acid by means of amide linkage, and this fatty acid contained unsaturated hydrocarbon chain. This hydrocarbon chain also contained aldehyde or hydroxyl functional group as substituent. Thus, ornithine residue-containing sclerotiotides are 12-membered ring structures. Furthermore, another sclerotiotide having lysine in the place of ornithine was also reported. Recently, from an Antarctica-sponge-derived *Aspergillus insulicola* HDN151418, three new sclerotiotides M–O (cyclic tripeptides) were discovered, whereby the variations in these three peptides were due to the nature of functional groups or substituents on the unsaturated hydrocarbon chain which is

linked to the α-amino group of the ornithine residue [81]. Thus, sclerotiotides are examples of backbone-sidechain cyclized peptides.

As already mentioned, cyclic peptides have been discovered from entomopathogenic fungi as well. For instance, hirsutide, a cyclic tetrapeptide of sequence, cyclo-(L-NMe-Phe-L-Phe-L-NMe-Phe-L-Val), was reported from a spider-derived entomopathogenic fungus, *Hirsutella* sp. in New Zealand [82]. Three new cyclic tetrapeptides whose sequences were somewhat similar to the hirsutide's sequence were identified from a fungus, *Onychocola sclerotica*, which was isolated from a poultry farm soil in Indonesia [83]. These three cyclic tetrapeptides were noted to block the cardiac calcium channels (Cav1.2), but not the hERG potassium channel [83]. Novel cyclic tetrapeptides, pseudoxylallemycins A–F, were discovered from a termite-associated fungus, *Pseudoxylaria* sp. X802, which was on the *Microtermes* sp. colony collected in South Africa [84]. The pseudoxylallemycins B–D possessed the unusual allenyl modification on the aromatic moiety.

Epichlicin, a cyclic octapeptide containing a β-amino acid residue, was isolated from an endophytic fungus *Epichloe typhina* of timothy plant (*Phleum pretense L.*) [85]. Epichlicin is rather a lipocyclic peptide as it possesses long hydrocarbon sidechain of the β-amino acid residue, viz., 3-amino tetradecanoic acid. Epichlicin was found to inhibit the spore germination of *Cladosporium phlei*, which is a pathogenic fungus of timothy plant. Recently, Ekanayake et al. had reported the identification of three cyclic octapeptides, broomeanamides from the fungus *Sphaerostilbella broomeana* (TFC201724) collected from the foothills of Himalayas in India [86]. Examples of a few fungal cyclic peptides possessing unusual amino acids are listed in Table 6.1.

Cyclosporin A is a very popular fungal cyclic peptide since it has been used as an immunosuppressive drug. Cyclosporin A has been identified in various fungal genera and species, e.g., *Tolypocladium* sp., *Fusarium* sp., *Aspergillus* sp., *Trichoderma* sp., and *Beauveria nivea* [9]. Several variants/analogs of cyclosporins have also been identified from different fungi. In addition to the immunosuppressant activity, cyclosporins also exhibit anti-inflammatory, anti-fungal, and anti-parasitic activities. Cyclosporin A and other analogues are composed of 11 amino acid residues, among which some are N-methylated residues (see Fig. 6.4).

6.2.3 Cyclic Depsipeptides

Cyclic depsipeptides (CDPs) are another class of NRPs that contain backbone ester (lactone) bond(s), due to hydroxy acid residue(s) amidst the conventional amide (peptide) units. Besides the hydroxy acid residue, many CDPs also contain N-methylated amino acid residues. About 350 different CDPs have been reported mainly from the genera *Acremonium*, *Aspergillus*, *Beauveria*, *Fusarium*, *Metarhizium*, *Alternaria*, etc. [88]. CDPs of different sizes have been discovered from various fungal species, albeit cyclic hexadepsipeptides (a hydroxy acid and five amino acid residues) represent the largest class of fungal CDPs that have been

Table 6.1 A few examples of fungal cyclic peptides possessing some unusual amino acid residues

S. no.	Unusual amino acid residue in cyclic peptide	Name of the cyclic peptide	Source	Biological activity (if any)	References
1a.	Anthranilic acid (ATA)	**Psychrophilins**[a]	*Penicillium* sp. (psychrotolerant) & *aspergillus* sp. (marine)	Psychrophilin D has moderate activity in the P388 murine leukemia cell assay	[71–73, 87]
1b.	ATA and N-methylated residues	**Cycloaspeptides A & D**	*Penicillium* sp. (psychrotolerant)	Moderate activity against *Plasmodium falciparum*	[71, 72]
		Versicotide C	*Aspergillus versicolor* (marine)	Not cytotoxic	[73]
2.	γ-Amino butyric acid (GABA)	**Unguisins**	*Emericella unguis* (marine)	–	[74, 75]
		Unguisin F	*Mucor irregularis* (endophyte from *Moringa stenopetala*)	–	[76]
3.	α-Amino isobutyric acid (Aib)	**Scytalidamides A & B**	*Scytalidium* (marine)	In vitro cytotoxic to human colon carcinoma tumor cell lines	[77]
4.	Dehydrotryptophan (Δ-Trp) & ATA	**Sclerotides**	*Aspergillus sclerotiorum* (marine)	–	[78, 79]
5.	Ornithine & N-methylated residues	**Sclerotiotides**[b]	*Aspergillus sclerotiorum* (marine) and *Aspergillus insulicola* (Antarctic sponge)	–	[80, 81]
6.	β-Amino acid residue (contains long hydrocarbon sidechain)	**Epichlicin** (Lipocyclic peptide)	*Epichloe typhina* (endophyte of timothy plant, *Phleum pretense L.*)	Inhibits spore germination of *Cladosporium phlei*, which is a pathogenic fungus of timothy	[85]

[a]Psychrophilins AD have a nitro group instead of an amino group, and the nitrogen atom of the indole group in the sidechain of tryptophan is involved in a peptide bond of the cyclic backbone

[b]Sclerotiotides are examples of backbone-sidechain cyclized peptides, whereby the sidechain amino group of ornithine participates in the formation of a peptide unit of the cyclic backbone

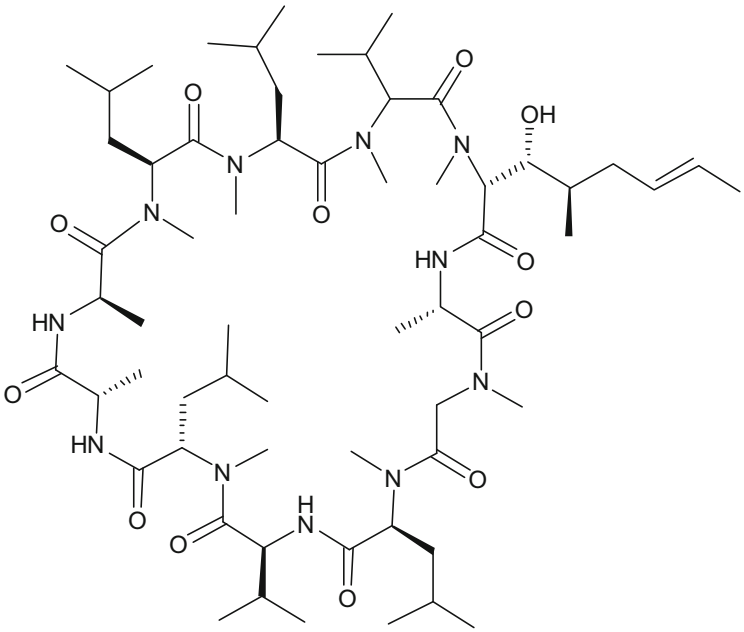

Fig. 6.4 Structure of cyclosporin A

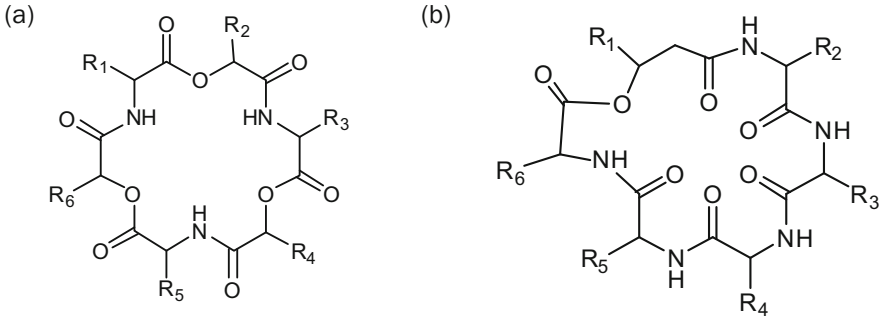

Fig. 6.5 Structure of cyclodepsipeptides with (**a**) α-hydroxyacid and (**b**) β-hydroxyacid

identified thus far [88]. CDPs may be classified into two categories based on the nature of hydroxy acid (HA): (i) α-HA-containing CDPs (α-HA CDPs) and (ii) β-HA-containing CDPs (β-HA CDPs). Molecular structural properties of some α-HA CDPs and a few β-HA CDPS are delineated herein.

α-HA CDPs Sansalvamide, beauvenniatins, allobeauvericins, beauvericins, enniatins, destruxins, roseotoxins, isaridins, etc. are some examples of CDPs that have α-HA in their sequences. The cyclohexadepsipeptides beauvenniatins, allobeauvericins, beauvericins, and enniatins are interesting in that they have three

ester and three amide bonds which are arranged in an alternating manner in their cyclic backbone structure (see Fig. 6.5a) [88]. Another intriguing aspect is that these four classes of CDPs have been identified from different fungal genera. Beauvenniatins have been identified from *Acremonium* sp., many beauvericins can be found in *Beauveria bassiana*, allobeauvericins were reported from *Paecilomyces tenuipes*, and enniatins have been discovered predominantly from *Fusarium* sp. [88]. A few beauvericins have been reported from *Paecilomyces tenuipes* also [88]. Enniatins have been found in endophytic as well as from entomogenous *Fusarium* sp. Likewise, though destruxins, roseotoxins, and isaridins have been identified from different fungal genera, these three classes may be grouped together based on the similarities in their molecular structures, wherein these peptides contain a β-amino acid residue (β-alanine) as part of their cyclic backbone, in addition to an α-HA (see Fig. 6.5b) [20, 89–91]. Destruxins are produced by the entomopathogenic *Metarhizium anisopliae* [89], roseotoxins are produced by *Trichothecium roseum* [90], while isaridins can be found in *Isaria* sp. [20, 91]. Thus, it is interesting to note that structurally very similar CDPs can be found from different fungal genera or species. In other words, fungi of different genera or species produce structurally similar CDPs. Perhaps, similar biosynthetic pathways involving NRPSs might be operating in different fungal genera.

The cyclohexadepsipeptides hirsutatins from the insect pathogenic *Hirsutella nivea* BCC 2594 and trichodepsipeptides from the filamentous fungus *Trichothecium* sp. can also be grouped in one class, although they are found in two different genera, since these peptides have identical cyclic backbone structure [92, 93]. Both hirsutatins and trichodepsipeptides contain two α-HA residues that are bonded next to each other and, hence, have two adjacent ester bonds in their cyclic backbone, albeit there are minor variations in the sidechains of one or two residues between hirsutatins and trichodepsipeptides. The leualacins reported from the fungus *Hapsidospora irregularis* are cyclic pentadepsipeptides, contain two α-HA residues (α-hydroxy leucine) that are not adjacent in its sequence, and have a β-amino acid residue, β-alanine [94].

The cyclic octadepsipeptide, bassionolide, and the "series of PF1022" CDPs have identical cyclic backbone architecture, which is characterized by equal number of ester and amide bonds occurring in an alternating fashion. The bassionolide was identified from *Beauveria bassiana* and *Verticillium lecanii* [95]. It is composed of four hydroxy valine and four N-methyl leucine residues, and hence, the sequence of bassionolide can be written as cyclo-[(N-Met Leu – Hyd. Val)$_4$]. The series of "PF1022" CDPs were identified in *Mycelia sterilia*, and all the peptides in this series possess four N-methyl leucine residues, whereas the α-HA residues in this series can be hydroxy alanine (Hyd. Ala) or hydroxy phenylalanine (Hyd. Phe) or hydroxy tyrosine (Hyd. Tyr) [96]. The PF1022F, cyclo-[(N-Met Leu – Hyd. Ala)$_4$] was also identified in *Trichoderma asperellum*, which was an endophyte of a traditional Chinese medicinal plant [97]. Thus, the four N-Met leucine residues are conserved across the sequences of bassionolide and all the CDPs of PF1022 series.

β-HA CDPs Beauveriolides, oryzamides, isariins, and emericellamides possess β-HA residue, which has somewhat long hydrocarbon sidechain on the β-carbon (with respect to the carboxyl group). Hence, these CDPs are also called cyclic lipopeptides. Beauverolides are tetra CDPs, while oryzamides, isariins, and emericellamides are cyclic hexadepsipeptides. Beauverolides are mainly present in *Beauveria* sp., especially in *B. bassiana* [98–100]. Oryzamides were identified from the sponge-derived fungus *Nigrospora oryzae* PF18 [101]. The CDP isariin was first reported by Vining and Taber way back in 1962 from *Isaria cretacea* [102]. Thereafter, Baute et al. (1981) and Deffieux et al. (1981) identified three variants of isariin (isariins B, C, and D) from *Isaria felina* [103, 104]. Based on LC-ESI-MS/MS and NMR spectroscopy, six more variants of isariin were identified, wherein these variants differed only at one particular amino acid residue that was bonded to the hydroxyl group of the β-HA [20]. Interestingly, a novel isariin (iso-isariin B) and a known isaridin (isaridin E) were discovered in *Beauveria felina* also [105]. Emericellamides were reported from *Aspergillus* [106] as well as from marine-derived *Emericella* sp. [107]. Once again it is notable that peptides having very similar molecular structures and identical cyclic backbone architecture are produced by fungi belonging to different genera, as evident in the cases of oryzamides, isariins, and emericellamides.

Halobacillin, a cyclic octadepsipeptide, was first reported from a marine *Bacillus*. However, it was also discovered in an endophytic fungus *Trichoderma asperellum* that was residing in a traditional Chinese medicinal plant [97]. Halobacillin consists of a β-HA residue having a hydrocarbon sidechain ($C_{12}H_{25}$) on the β-carbon, and hence, it is also an example of cyclic lipopeptide. Examples of a few fungal cyclic depsipeptides are listed in Table 6.2.

6.3 Ribosomal Fungal Peptides

6.3.1 Disulfide-Bonded Peptides: Fungal Defensins

Disulfide-bonded peptides are also a type of cyclic peptides, whereby the formation of cyclic or ring-like structure involves the sidechain atoms rather than the backbone atoms. In other words, disulfide-bonded peptides come under the class of sidechain-sidechain cyclized peptides. Defensins are disulfide-bonded (cysteine-rich) peptides that have been identified in vertebrates including humans, in plants, as well as in insects [108–112]. These polypeptides constitute an important class of antimicrobial peptides (AMPs), which has a vital role as part of the innate immune system, and thus contribute to the host defense against bacterial, fungal, and viral infections [109]. When compared to the total number of defensins known from vertebrates, plants, and insects, the number of defensins or defensin-like peptides (fDLPs) identified from fungi is relatively less [113]. Nevertheless, fDLPs are touted to be of therapeutic value particularly in the context of rise in the drug-resistant strains, e.g., a derivative of a fDLP, NZ2114, could reach the preclinical stage of drug discovery [114].

Table 6.2 A few examples of fungal cyclic depsipeptides

α -HA-containing CDPs			
I. CDPs having one α-HA only			
S. No.	**Names of the CDPs**	**Sources**	**References**
1.	Destruxins[a]	*Metarhizium anisopliae*	[89]
2.	Roseotoxins[a]	*Trichothecium roseum*	[90]
3.	Isaridins[a]	*Isaria* sp.	[20, 91]
II. CDPs having more than one α-HA			
S. No.	**Names of the CDPs**	**Sources**	**References**
1.	Beauvenniatins[b]	*Acremonium* sp.	[88]
2.	Allobeauvericins[b]	*Paecilomyces tenuipes*	[88]
3.	Beauvericins[b]	*Beauveria bassiana*	[88]
4.	Enniatins[b]	*Fusarium* sp.	[88]
5.	Hirsutatins	*Hirsutella nivea*	[88]
6.	Trichodepsipeptides	*Trichothecium* sp.	[88]
β-HA-containing CDPs[c]			
S. No.	**Names of the CDPs**	**Sources**	**References**
1.	Beauverolides	*Beauveria* sp.	[98–100]
2.	Isariins	*Isaria* sp.	[20, 102–104]
3.	Oryzamides	*Nigrospora oryzae*	[101]
4.	Emericellamides	*Emericella* sp. *Aspergillus*	[106, 107]

[a]These CDPs also contain a β-amino acid
[b]α-HAs and α-amino acids in these CDPs are bonded in an alternating manner, and hence, the cyclic backbone is made up of alternating ester and amide bonds, in these CDPs
[c]In these CDPs, the sidechain of β-HA contains longer hydrocarbon chain, and therefore, these CDPs are also referred to as **cyclic lipopeptides**

Plectasin is the first fungal defensin isolated from a saprophytic ascomycete, *Pseudoplectania nigrella* [115]. The in vitro activity of this peptide against *Streptococcus pneumoniae* was comparable to that of penicillin and vancomycin. The recombinantly produced plectasin was found to be active even against the antibiotic-resistant strains of *S. pneumoniae*. It exhibits antimicrobial activity by directly binding to the bacterial cell wall precursor lipid II, and the stoichiometry of this binding was observed to be equimolar [116]. Plectasin is composed of 40 amino acid residues and contains 3 disulfide bonds (UniProt KB ID: Q53I06). The sequence of plectasin showed 50–55% identity with sequences of several invertebrate defensins, whereas it did not have any significant similarity to the mammalian α- and β-defensin sequences [115]. The 3D molecular structure of plectasin contains an α-helix and two antiparallel β-strands stabilized by three disulfide bonds. By considering the sequence together with the 3D molecular structural features, it was evident that plectasin indeed belonged to the defensin family [115]. A derivative of plectasin, NZ2114 was found to be potent not only against the multiply drug-resistant bacterial strains in vitro, but it also was efficacious in the in vivo pharmacodynamic investigation carried out in a murine infection model [2]. Furthermore,

NZ2114 could also exhibit strong bactericidal activity in the cerebrospinal fluid (CSF) by penetrating into the CSF in an experimental meningitis model, indicating its potential to treat infections in the central nervous system (CNS), including the penicillin-resistant pneumococcal meningitis [117].

Eurocin is another new fungal defensin isolated from another ascomycete *Eurotium amstelodami* [118]. It is a 42-amino acid residue-long polypeptide with three disulfide bonds. Its 3D molecular structure is made up of an α-helix and two β-strands forming an antiparallel β-sheet (*c.f.* PDB ID 2LT8), referred to as cysteine-stabilized αβ-fold (CSαβ) [119, 120]. Thus, the eurocin's 3D structure was found to be highly homologous to the structures reported from other fungal and invertebrate defensins. Similar to plectasin, eurocin shows more strong effect on Gram-positive bacteria than Gram-negative bacteria. From the cell-free assay experiments, it was observed that eurocin also binds to the bacterial cell wall precursor lipid II, suggesting that both eurocin and plectasin follow a similar binding mechanism. In vivo and in vitro antimicrobial assays showed that eurocin is a fast and effective antibiotic against *Streptococci* even at low concentrations.

Copsin is a novel antibiotic identified in the mushroom *Coprinopsis cinerea*, and perhaps, it is the first defensin to be identified from the fungal phylum of *Basidiomycota* [121]. It is composed of 57 amino acid residues with N-terminal pyroglutamic acid and 6 disulfide bonds. The sequence of copsin showed 20–27% identity with the defensins from other invertebrates, fungi, and plants. The 3D molecular structure of eurocin also consists of CSαβ fold, similar to the structures of eurocin and plectasin (vide supra). The sequence of the secondary structural elements displayed high identity to the known defensins that possessed CSαβ structural motif; however, the length and the composition of the loop regions and the termini were found to be unique. Copsin inhibited Gram-positive bacteria such as *Bacillus subtilis*, *Listeria* sp., and *Enterococcus* sp. in the low μg/ml range, and it showed most potent activity against *Listeria monocytogenes*, which is a well-known food-borne pathogen. Copsin did not elicit antibacterial activity, when treated with reducing agent (e.g., dithiothreitol, DTT), indicating the importance of disulfide bonds for the integrity of its 3D structure and its biological function.

The defensin micasin identified from a dermatophytic fungus *Microsporum canis* was found to act against both Gram-positive and Gram-negative bacteria suggesting that the micasin's mode of antimicrobial action is different from eurocin and plectasin [122]. Micasin could also kill two clinical isolates of methicillin-resistant *Staphylococcus aureus* and the opportunistic pathogen *Pseudomonas aeruginosa* at low micromolar concentrations. Very recently, a non-hemolytic fDLP, Pyronesin 4 (Py4), was reported from the basal filamentous ascomycete *Pyronema confluens* [113]. Py4 inhibited the bacterial cell wall biosynthesis, and it was found to be highly stable in the mammalian serum.

A brief survey into UniProt KB database looking for "fungal defensins" revealed a few more peptides. Though only a few fungal defensins have been isolated and characterized, six different families of fDLPs have been identified from the available fungal genome sequences through computational prediction, whereby the

computational analysis indicated very good conservation of three different types of defensins between animals and fungi [119].

6.3.2 Fungal RiPPs

α-Amanitin, phallacidin, ustiloxin, phomopsin, and epichloëcyclin are a few examples of fungal RiPPs [5]. Hydroxylation, methylation, epimerization, and acetylation are some posttranslational modifications that have been observed on fungal RiPPs [3]. As reviewed by Vogt and Kunzler, the fungal RiPPs identified thus far have been classified into four different families: amatoxins/phallotoxins, borosins, dikaritins, and epichloëcyclins. α-Amanitin and phallacidin represent the first fungal RiPP family, identified from the poisonous mushrooms belonging to the genus *Amanita* [123]. α-Amanitin (amatoxin) is a bicyclic octapeptide, while the related phallacidin (phallotoxin) is a bicyclic heptapeptide [123]. Amatoxins inhibit RNA polymerase II, and amatoxin poisoning can affect the liver, leading to death as well; however, phallotoxins elicit toxicity by stabilizing F-actin [123]. The phallotoxins exhibit toxicity only through parenteral administration and not orally due to poor absorption [123].

The family dikaritins constitutes ustiloxins, phomposins, and asperipins. These three classes of peptides were identified from ascomycetes. A striking feature in the molecular structures of dikaritins is the presence of "ether" linkage as part of the cyclic/ring structure involving the sidechain of tyrosine [5]. Ustiloxins and phomposins inhibit tubulin polymerization and suppress mitosis, whereas the biological activity of asperipin is not yet known [5]. The borosin family consists of cyclic peptides called "omphalotins" that have N-methylation at several amide bonds [124]. Prior to the identification of omphalotins, the N-methylation was mostly thought to be prevalent in non-ribosomal peptides only. Omphalotins E–I from the basidiomycete *Omphalotus olearius* have been reported to have nematicidal activity [124]. The endophytic fungus belonging to the genus *Epichloë* synthesizes cyclic nonapeptides called epichloëcyclins, whose biological function is not reported yet [125].

6.4 Fungal Peptides as Drugs

Caspofungin (brand name: "Cancidas") is a semi-synthetic lipopeptide, which is the first member in the new drug class called "echinocandins," as named by Merck & Co., Inc. (https://go.drugbank.com/drugs/DB00520). It was originally obtained as a fermentation product from the fungus *Glarea lozoyensis* [126]. Many clinical trials proved "caspofungin" to be efficacious for the treatment of esophageal, oropharyngeal, and invasive candidiasis as well as for invasive aspergillosis [127, 128]. It was approved by the Food and Drug Administration (FDA) for use in the United States [127, 129]. A few other members of echinocandin class are (i) micafungin (brand name: "Micamine"; Drug Bank Accession No. DB01141) and

(ii) anidulafungin (brand name: "Ecalta/Eraxis"; Drug Bank Accession No. DB00362) [129]. Thus, caspofungin, micafungin, and anidulafungin became the "first-line" antifungal agents to treat invasive candidiasis. Resistance towards the echinocandin drugs also has been observed [130]. However, it was recently reported that caspofungin elicits antifungal activity even against the multidrug-resistant (MDR) *Candida*, when the drug was prepared in low ionic solutions [131]. The molecular structures of these three lipopeptides have cyclic peptidic backbone comprising six amide (peptide) bonds, and hence, these three are cyclic hexapeptides [9].

The cyclic undecapeptide cyclosporin A (CsA) is popularly known for its immunosuppressive activity [132]. Due to its selective immunosuppressant activities, CsA could be successfully applied in many transplantation therapies, and thereby significant improvement was observed in the survival rates after the grafting of the solid organs [132]. CsA in combination with other suitable drug has proven to be good for treating rheumatoid arthritis as well [133, 134]. Although CsA was identified from various fungal sources such as *Aspergillus* sp., *Beauveria nivea*, *Fusarium oxysporum*, and *Trichoderma polysporum*, the fungus *Tolypocladium inflatum* has been extensively utilized for the production of CsA by following different fermentation techniques [9, 132]. The other biological functions of CsA include antifungal, antiparasitic, and anti-inflammatory [132]. About 25 different analogues of cyclosporin have been identified, which have also been observed to have immunosuppressant effect as well as antifungal activity [9]. Cyclosporin is sold under different brand names, e.g., Sandimmune, Cequa, Gengraf, etc. (https://go.drugbank.com/drugs/DB00091).

6.5 Concluding Remarks

Because of the continuous emergence of resistant strains of pathogens towards various drug compounds, the search for novel antibiotics and antimicrobial compounds has not stopped for several years from now. Particularly the peptide-based antibiotics and antimicrobial peptides have been shown to elicit potent activity even against drug-resistant microbial strains [135, 136]. Therefore, exploration of peptide-based drugs and antimicrobials have become more preferable. The discussions in the previous sections clearly indicate that fungi are indeed very valuable (re)sources for exploring and identifying novel peptide antibiotics. Decades of research investigations on various fungal sources have unearthed numerous non-ribosomal peptides leading to creation of a repertoire of knowledge on these peptides. Therefore, the non-ribosomal fungal peptides have been tried and tested a lot for developing therapeutic compounds, for example, the echinocandin and the cyclosporin classes of drugs (see Sect. 6.4) [137]. Identification of cytotoxic fungal peptaibols and their synthetic analogues, e.g., gichigamins from *Tolypocladium* species in Michigan and lipopeptaibols from a Himalayan *Trichoderma* species, provides encouraging signs for development of novel anti-cancer or anti-tumor drugs [16, 17, 28]. Despite this level of progress, several studies are still ongoing,

and many investigations need to be carried out in future, in order to obtain improved and clearer understanding about their natural role(s) of non-ribosomal fungal peptides [138]. In contrast, RiPPs from fungi have been reported only in the recent past. Therefore, there is enormous scope to investigate the potential of fungal RiPPs, so as to develop them into drugs of therapeutic value and make them widely available in the market. The discovery of fungal RiPPs might stimulate the interest towards "fungal sources" for searching peptide-based therapeutics [5]. Altogether, in addition to their natural multiple vital roles in various contexts in the ecosystem, fungi are indeed valuable resources for drug discovery and for various agricultural applications. Thus, the rise of fungal-based products having diverse applications towards general human welfare seems imminent, in the near future.

References

1. Niu X, Thaochan N, Hu Q (2020) Diversity of linear non-ribosomal peptide in biocontrol fungi. J Fungi (Basel) 6(2):61. https://doi.org/10.3390/jof6020061
2. Andes D, Craig W et al (2009) *In vivo* pharmacodynamic characterization of a novel plectasin antibiotic, NZ2114, in a murine infection model. Antimicrob Agents Chemother 53(7): 3003–3009. https://doi.org/10.1128/AAC.01584-08
3. Arnison PG, Bibb MJ et al (2013) Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature. Nat Prod Rep 30(1):108–160. https://doi.org/10.1039/c2np20085f
4. Dang T, Süssmuth RD (2017) Bioactive peptide natural products as lead structures for medicinal use. Acc Chem Res 50(7):1566–1576. https://doi.org/10.1021/acs.accounts.7b00159
5. Vogt E, Künzler M (2019) Discovery of novel fungal RiPP biosynthetic pathways and their application for the development of peptide therapeutics. Appl Microbiol Biotechnol 103(14): 5567–5581. https://doi.org/10.1007/s00253-019-09893-x
6. Kleinkauf H, Von Döhren H (1996) A nonribosomal system of peptide biosynthesis. Eur J Biochem 236:335–351. https://doi.org/10.1111/j.1432-1033.1996.00335.x
7. Marahiel MA, Stachelhaus T, Mootz HD (1997) Modular peptide synthetases involved in nonribosomal peptide synthesis. Chem Rev 97:2651–2673. https://doi.org/10.1021/cr960029e
8. Alonzo DA, Schmeing TM (2020) Biosynthesis of depsipeptides, or Depsi: the peptides with varied generations. Protein Sci 29(12):2316–2347. https://doi.org/10.1002/pro.3979
9. Wang X, Lin M et al (2017) Structural diversity and biological activities of fungal cyclic peptides, excluding cyclodipeptides. Molecules 22(12):2069. https://doi.org/10.3390/molecules22122069
10. Youssef FS, Ashour ML et al (2019) A comprehensive review of bioactive peptides from marine fungi and their biological significance. Mar Drugs 17(10):559. https://doi.org/10.3390/md17100559
11. Brückner H, Przybylski M (1984) Isolation and structural characterization of polypeptide-antibiotics of the peptaibol class by high-performance liquid chromatography with field desorption and fast atom bombardment mass spectrometry. J Chromatogr 296:263–275
12. Pandey RC, Cook JC Jr, Rinehart KL Jr (1977) High resolution and field desorption mass spectrometry studies and revised structures of alamethicins I and II. J Am Chem Soc 99:8469–8483. https://doi.org/10.1021/ja00468a016
13. Pandey RC, Cook JC Jr, Rinehart KL Jr (1977) Reptaibophol antibiotics. 2. Structures of the peptide antibiotics emerimicins III and IV. J Am Chem Soc 99:5205–5206. https://doi.org/10.1021/ja00457a064

14. Pandey RC, Meng H et al (1977) Structure of antiamoebin I from high resolution field desorption and gas chromatographic mass spectrometry studies. J Am Chem Soc 99:5203–5205. https://doi.org/10.1021/ja00457a063
15. Ruiz N, Wielgosz-Collin G et al (2007) New Trichobrachins, 11-residue peptaibols from a marine strain of *Trichoderma longibrachiatum*. Peptides 28:1351–1358. https://doi.org/10.1016/j.peptides.2007.05.012
16. Singh VP, Pathania AS et al (2020) 14-residue peptaibol velutibol A from *Trichoderma velutinum*: its structural and cytotoxic evaluation. RSC Adv 10:31233–31242. https://doi.org/10.1039/d0ra05780k
17. Singh VP, Yedukondalu N et al (2018) Lipovelutibols A–D: cytotoxic lipopeptaibols from the Himalayan cold habitat fungus *Trichoderma velutinum*. J Nat Prod 81:219–226. https://doi.org/10.1021/acs.jnatprod.6b00873
18. Jaworski A, Brückner H (1999) Detection of new sequences of peptaibol antibiotics trichotoxins A-40 by on-line liquid chromatography-electrospray ionization mass spectrometry. J Chromatogr A 862:179–189. https://doi.org/10.1016/S0021-9673(99)00931-0
19. Poirier L, Amiard JC et al (2007) Determination of peptaibol trace amounts in marine sediments by liquid chromatography/electrospray ionization-ion trap-mass spectrometry. J Chromatogr A 1160:106–113. https://doi.org/10.1016/j.chroma.2007.04.006
20. Sabareesh V, Ranganayaki RS et al (2007) Identification and characterization of a library of microheterogenous cyclohexadepsipeptides from the fungus *Isaria*. J Nat Prod 70(5):715–729
21. Stoppacher N, Reithner B et al (2007) Profiling of trichorzianines in culture samples of *Trichoderma atroviride* by liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 21:3963–3970
22. Van Bohemen AI, Zalouk-Vergnoux A et al (2016) Development and validation of LC-MS methods for peptaibol quantification in fungal extracts according to their lengths. J Chromatogr B Analyt Technol Biomed Life Sci 1009-1010:25–33. https://doi.org/10.1016/j.jchromb.2015.11.039
23. Toniolo C, Bruckner H (2007) Peptaibiotics. Chem Biodivers 4:1021–1022. https://doi.org/10.1002/cbdv.200790093
24. Krause C, Kirschbaum J, Brückner H (2006) Peptaibiomics: an advanced, rapid and selective analysis of peptaibiotics/peptaibols by SPE/LC-ES-MS. Amino Acids 30:435–443. https://doi.org/10.1007/s00726-005-0275-9
25. Daniel JF, Filho ER (2007) Peptaibols of trichoderma. Nat Prod Rep 24(5):1128–1141. https://doi.org/10.1039/b618086h
26. Degenkolb T, Berg A et al (2003) The occurrence of peptaibols and structurally related peptaibiotics in fungi and their mass spectrometric identification via diagnostic fragment ions. J Pept Sci 9:666–678. https://doi.org/10.1002/psc.497
27. Raap J, Erkelens K et al (2005) Fungal biosynthesis of non-ribosomal peptide antibiotics and α, α-dialkylated amino acid constituents. J Pept Sci 11:331–338. https://doi.org/10.1002/psc.621
28. Du L, Risinger AL et al (2017) Unique amalgamation of primary and secondary structural elements transform peptaibols into potent bioactive cell-penetrating peptides. Proc Natl Acad Sci U S A 114:E8957–E8966. https://doi.org/10.1073/PNAS.1707565114
29. Hao X, Li S et al (2021) Acremopeptaibols A-F, 16-residue peptaibols from the sponge-derived *Acremonium* sp. IMB18-086 cultivated with heat-killed *Pseudomonas aeruginosa*. J Nat Prod 84(11):2990–3000. https://doi.org/10.1021/acs.jnatprod.1c00834
30. Jiao WH, Khalil Z et al (2018) Trichodermides A-E: new peptaibols isolated from the Australian termite nest-derived fungus *Trichoderma virens* CMB-TN16. J Nat Prod 81(4):976–984. https://doi.org/10.1021/acs.jnatprod.7b01072
31. Stoppacher N, Neumann NKN et al (2013) The comprehensive peptaibiotics database. Chem Biodivers 10:734–743. https://doi.org/10.1002/cbdv.201200427

32. Zhang SH, Yang J et al (2021) Longibramides A-E, peptaibols isolated from a mushroom derived fungus *Trichoderma longibrachiatum* Rifai DMG-3-1-1. Chem Biodivers 18(5): e2100128. https://doi.org/10.1002/cbdv.202100128
33. Mohamed-Benkada M, Montagu M et al (2006) New short peptaibols from a marine *Trichoderma* strain. Rapid Commun Mass Spectrom 20:1176–1180. https://doi.org/10.1002/rcm.2430
34. Mohamed-Benkada M, François Pouchus Y et al (2016) Identification and biological activities of long-chain peptaibols produced by a marine-derived strain of *Trichoderma longibrachiatum*. Chem Biodivers 13(5):521–530. https://doi.org/10.1002/cbdv.201500159
35. van Bohemen AI, Ruiz N et al (2021) Pentadecaibins I-V: 15-residue peptaibols produced by a marine-derived *Trichoderma* sp. of the *Harzianum* clade. J Nat Prod 84(4):1271–1282. https://doi.org/10.1021/acs.jnatprod.0c01355
36. Balaram P, Krishna K et al (1992) The properties of ion channels formed by zervamicins. Eur Biophys J 21:117–128. https://doi.org/10.1007/BF00185426
37. Nagaraj R, Balaram P (1981) Alamethicin, a Transmembrane Channel. Acc Chem Res 14: 356–362. https://doi.org/10.1021/ar00071a005
38. Sansom MSP (1993) Structure and function of channel-forming peptaibols. Q Rev Biophys 26:365–421
39. Mueller P, Rudin DO (1968) Action potentials induced in biomolecular lipid membranes. Nature 217:713–719. https://doi.org/10.1038/217713a0
40. Fox RO, Richards FM (1982) A voltage-gated ion channel model inferred from the crystal structure of alamethicin at 1.5-Å resolution. Nature 300:325–330. https://doi.org/10.1038/300325a0
41. Auvin-Guette C, Rebuffat S et al (1992) Trichogin A IV, an 11-residue lipopeptaibol from *Trichoderma longibrachiatum*. J Am Chem Soc 114:2170–2174. https://doi.org/10.1021/ja00032a035
42. Gurunath R, Balaram P (1995) A nonhelical, multiple beta-turn conformation in a glycine-rich heptapeptide fragment of Trichogin A IV containing a single central alpha-aminoisobutyric acid residue. Biopolymers 35:21–29. https://doi.org/10.1002/bip.360350104
43. Iqbal M, Balaram P (1981) Membrane channel forming polypeptides. 270-MHz Hydrogen-1 nuclear magnetic resonance studies on the conformation of the 11–21 fragment of suzukacillin. Biochemistry 20:4866–4871. https://doi.org/10.1021/bi00520a010
44. Iqbal M, Balaram P (1981) The 3_{10} helical conformation of the amino terminal decapeptide of suzukacillin. 270MHz ^{1}H NMR evidence for eight intramolecular hydrogen bonds. J Am Chem Soc 103:5548–5552. https://doi.org/10.1021/ja00408a045
45. Karle IL, Flippen-Anderson J et al (1987) Conformation of a 16-residue zervamicin IIA analog peptide containing three different structural features: 3(10)-helix, alpha-helix, and beta-bend ribbon. Proc Natl Acad Sci U S A 84:5087–5091. https://doi.org/10.1073/pnas.84.15.5087
46. Karle IL, Flippen-Anderson JL et al (1991) Crystal structure of [Leu1] zervamicin, a membrane ion-channel peptide: implications for gating mechanisms. Proc Natl Acad Sci U S A 88: 5307–5311. https://doi.org/10.1073/pnas.88.12.5307
47. Karle IL, Perozzo MA et al (1998) Crystal structure of the channel-forming polypeptide antiamoebin in a membrane-mimetic environment. Proc Natl Acad Sci U S A 95:5501–5504. https://doi.org/10.1073/pnas.95.10.5501
48. Karle IL, Sukumar M, Balaram P (1986) Parallel packing of alpha-helices in crystals of the zervamicin IIA analog Boc-Trp-Ile-Ala-Aib-Ile-Val-Aib-Leu-Aib-Pro-OMe.2H2O. Proc Natl Acad Sci U S A 83:9284–9288. https://doi.org/10.1073/pnas.83.24.9284
49. Rebuffat S, Prigent Y et al (1991) Tricholongins BI and BII, 19-residue peptaibols from *Trichoderma longibrachiatum*. Solution structure from two-dimensional NMR spectroscopy. Eur J Biochem 201:661–674
50. Snook CF, Woolley GA et al (1998) The structure and function of antiamoebin I, a proline-rich membrane-active polypeptide. Structure 6:783–792. https://doi.org/10.1016/S0969-2126(98)00079-3

51. Aravinda S, Shamala N, Balaram P (2008) Aib residues in peptaibiotics and synthetic sequences: analysis of nonhelical conformations. Chem Biodivers 5(7):1238–1262. https://doi.org/10.1002/cbdv.200890112
52. Chugh JK, Wallace BA (2001) Peptaibols: models for ion channels. Biochem Soc Trans 29(4): 565–570. https://doi.org/10.1042/bst0290565
53. Whitmore L, Wallace BA (2004) Analysis of peptaibol sequence composition: implications for in vivo synthesis and channel formation. Eur Biophys J 33(3):233–237. https://doi.org/10.1007/s00249-003-0348-1
54. Auvin-Guette C, Rebuffat S et al (1993) Structural elucidation of Trikoningins KA and KB, peptaibols from *Trichoderma koningii*. J Chem Soc Perkin Trans 1:249–255
55. Rowley DC, Kelly S et al (2003) Halovirs A-E, new antiviral agents from a marine-derived fungus of the genus *Scytalidium*. Bioorg Med Chem 11:4263–4274. https://doi.org/10.1016/S0968-0896(03)00395-X
56. Momose I, Onodera T et al (2019) Leucinostatin Y: a peptaibiotic produced by the entomoparasitic fungus *Purpureocillium lilacinum* 40-H-28. J Nat Prod 82:1120–1127. https://doi.org/10.1021/acs.jnatprod.8b00839
57. Gupta S, Krasnoff SB et al (1991) Structures of the efrapeptins: potent inhibitors of mitochondrial ATPase from the fungus *Tolypocladium niveum*. J Am Chem Soc 113:707–709. https://doi.org/10.1021/ja00002a068
58. Gupta S, Krasnoff SB et al (1992) Structure of efrapeptins from the fungus *Tolypocladium niveum*: peptide inhibitors of mitochondrial ATPase. J Org Chem 57:2306–2313. https://doi.org/10.1021/jo00034a022
59. Uma MV, Sudha R, Balaram P (2001) Spermidine as a potential biosynthetic precursor to the 1,5-diazabicyclo[4:3:0]nonene residue in the efrapeptins. J Pept Res 58:375–379. https://doi.org/10.1034/j.1399-3011.2001.00915.x
60. Przybylski M, Dietrich I et al (1984) Elucidation of structure and microheterogeneity of the polypeptide antibiotics paracelsin and trichotoxin A-50 by fast atom bombardment mass spectrometry in combination with selective in situ hydrolysis. Biomed Mass Spectrom 11: 569–582
61. Rinehart KL Jr, Gaudioso LA et al (1981) Structures of eleven zervamicin and two emerimicin peptide antibiotics studied by fast atom bombardment mass spectrometry. J Am Chem Soc 103:6517–6520
62. Pócsfalvi G, Ritieni A et al (1997) Microheterogeneity characterization of a Paracelsin mixture from *Trichoderma reesei* using high-energy collision-induced dissociation tandem mass spectrometry. Rapid Commun Mass Spectrom 11:922–930
63. Sabareesh V, Balaram P (2006) Tandem electrospray mass spectrometric studies of proton and sodium ion adducts of neutral peptides with modified N- and C-termini: synthetic model peptides and microheterogeneous peptaibol antibiotics. Rapid Commun Mass Spectrom 20: 618–628. https://doi.org/10.1002/rcm.2349
64. Das MK, Raghothama S, Balaram P (1986) Membrane channel forming polypeptides. Molecular conformation and mitochondrial uncoupling activity of antiamoebin, an alpha-aminoisobutyric acid containing peptide. Biochemistry 25:7110–7117. https://doi.org/10.1021/bi00370a053
65. Mathew MK, Nagaraj R, Balaram P (1981) Alamethicin and synthetic peptide fragments as uncouplers of mitochondrial oxidative phosphorylation. Effect of chain length and charge. Biochem Biophys Res Commun 98:548–555. https://doi.org/10.1016/0006-291x(81)90875-5
66. Cross RL, Kohlbrenner WE (1978) The mode of inhibition of oxidative phosphorylation by efrapeptin (A23871). Evidence for an alternating site mechanism for ATP synthesis. J Biol Chem 253(14):4865–4873
67. Wada S, Iida A et al (1997) Role of the Gln/Glu residues of trichocellins A-II/B-II in ion-channel formation in lipid membranes and catecholamine secretion from chromaffin cells. Biochim Biophys Acta 1325(2):209–214. https://doi.org/10.1016/s0005-2736(96)00260-x

68. Hosotani N, Kumagai K et al (2007) SPF-5506-A4, a new peptaibol inhibitor of amyloid beta-peptide formation produced by *Trichoderma* sp. J Antibiot (Tokyo) 60(3):184–190. https://doi.org/10.1038/ja.2007.20
69. Hayakawa Y, Hattori Y et al (2008) Efrapeptin J, a new down-regulator of the molecular chaperone GRP78 from a marine *Tolypocladium* sp. J Antibiot (Tokyo) 61(6):365–371. https://doi.org/10.1038/ja.2008.51
70. Ghosh R, Noori MT, Ghangrekar MM (2017) Novel application of peptaibiotics derived from *Trichoderma* sp. for methanogenic suppression and enhanced power generation in microbial fuel cells. RSC Adv 7:10707–10717. https://doi.org/10.1039/C6RA27763B
71. Dalsgaard PW, Larsen TO et al (2004) Psychrophilin A and cycloaspeptide D, novel cyclic peptides from the psychrotolerant fungus *Penicillium ribeum*. J Nat Prod 67:878–881. https://doi.org/10.1021/np0303714
72. Dalsgaard PW, Larsen TO, Christophersen C (2005) Bioactive cyclic peptides from the psychrotolerant fungus *Penicillium algidum*. J Antibiot (Tokyo) 58(2):141–144. https://doi.org/10.1038/ja.2005.16
73. Peng J, Gao H et al (2014) Psychrophilins E-H and versicotide C, cyclic peptides from the marine-derived fungus *Aspergillus versicolor* ZLN-60. J Nat Prod 77(10):2218–2223. https://doi.org/10.1021/np500469b
74. Malmstrøm J (1999) Unguisins A and B: new cyclic peptides from the marine-derived fungus *Emericella unguis*. J Nat Prod 62(5):787–789. https://doi.org/10.1021/np980539z
75. Malmstrøm J, Ryager A et al (2002) Unguisin C, a GABA-containing cyclic peptide from the fungus *Emericella unguis*. Phytochemistry 60(8):869–872. https://doi.org/10.1016/s0031-9422(02)00150-4
76. Akone SH, Daletos G et al (2016) Unguisin F, a new cyclic peptide from the endophytic fungus *Mucor irregularis*. Z Naturforsch C J Biosci 71(1–2):15–19. https://doi.org/10.1515/znc-2015-0137
77. Tan LT, Cheng XC et al (2003) Scytalidamides A and B, new cytotoxic cyclic heptapeptides from a marine fungus of the genus *Scytalidium*. J Org Chem 68(23):8767–8773. https://doi.org/10.1021/jo030191z
78. Zheng J, Zhu H et al (2009) Novel cyclic hexapeptides from marine-derived fungus, *Aspergillus sclerotiorum* PT06-1. Org Lett 11(22):5262–5265. https://doi.org/10.1021/ol902197z
79. Long J, Chen Y et al (2021) Cyclic peptides from the soft coral-derived fungus *Aspergillus sclerotiorum* SCSIO 41031. Mar Drugs 19(12):701. https://doi.org/10.3390/md19120701
80. Zheng J, Xu Z et al (2010) Cyclic tripeptides from the halotolerant fungus *Aspergillus sclerotiorum* PT06-1. J Nat Prod 73(6):1133–1137. https://doi.org/10.1021/np100198h
81. Sun C, Zhang Z et al (2020) Antibacterial cyclic tripeptides from Antarctica-sponge-derived fungus *Aspergillus insulicola* HDN151418. Mar Drugs 18(11):532. https://doi.org/10.3390/md18110532
82. Lang G, Blunt JW, Cummings NJ, Cole AL, Munro MH (2005) Hirsutide, a cyclic tetrapeptide from a spider-derived entomopathogenic fungus, *Hirsutella* sp. J Nat Prod 68(8):1303–1305. https://doi.org/10.1021/np0501536
83. Pérez-Victoria I, Martín J et al (2012) Isolation and structural elucidation of cyclic tetrapeptides from *Onychocola sclerotica*. J Nat Prod 75(6):1210–1214. https://doi.org/10.1021/np3000987
84. Guo H, Kreuzenbeck NB et al (2016) Pseudoxylallemycins A-F, cyclic tetrapeptides with rare Allenyl modifications isolated from *Pseudoxylaria* sp. X802: a competitor of fungus-growing termite cultivars. Org Lett 18(14):3338–3341. https://doi.org/10.1021/acs.orglett.6b01437
85. Seto Y, Takahashi K et al (2007) Novel cyclic peptide, epichlicin, from the endophytic fungus, *Epichloe typhina*. Biosci Biotechnol Biochem 71(6):1470–1475. https://doi.org/10.1271/bbb.60700
86. Ekanayake DI, Perlatti B et al (2021) Broomeanamides: cyclic octapeptides from an isolate of the Fungicolous ascomycete *Sphaerostilbella broomeana* from India. J Nat Prod 84(7):2028–2034. https://doi.org/10.1021/acs.jnatprod.1c00414

87. Dalsgaard PW, Blunt JW et al (2004) Psychrophilin B and C: cyclic nitropeptides from the psychrotolerant fungus *Penicillium rivulum*. J Nat Prod 67(11):1950–1952. https://doi.org/10.1021/np0497954
88. Wang X, Gong X et al (2018) Structural diversity and biological activities of cyclic depsipeptides from fungi. Molecules 23(1):169. https://doi.org/10.3390/molecules23010169
89. Jegorov A, Havlícek V, Sedmera P (1998) Rapid screening of destruxins by liquid chromatography/mass spectrometry. J Mass Spectrom 33(3):274–280. https://doi.org/10.1002/(SICI)1096-9888(199803)33:3<274::AID-JMS630>3.0.CO;2-R
90. Jegorov A, Paizs B et al (2003) Profiling of cyclic hexadepsipeptides roseotoxins synthesized in vitro and in vivo: a combined tandem mass spectrometry and quantum chemical study. Eur J Mass Spectrom (Chichester) 9(2):105–116. https://doi.org/10.1255/ejms.531
91. Ravindra G, Ranganayaki RS et al (2004) Two novel hexadepsipeptides containing several modified amino acid residues from the fungus *Isaria*. Chem Biodivers 1:489–504
92. Isaka M, Palasarn S et al (2005) Cyclohexadepsipeptides from the insect pathogenic fungus *Hirsutella nivea* BCC 2594. J Nat Prod 68(11):1680–1682. https://doi.org/10.1021/np050246n
93. Sy-Cordero AA, Graf TN et al (2011) Cyclodepsipeptides, sesquiterpenoids, and other cytotoxic metabolites from the filamentous fungus *Trichothecium* sp. (MSX 51320). J Nat Prod 74(10):2137–2142. https://doi.org/10.1021/np2004243
94. Zhang S, Qiu Y et al (2017) Identification of cyclic depsipeptides and their dedicated synthetase from *Hapsidospora irregularis*. J Nat Prod 80(2):363–370. https://doi.org/10.1021/acs.jnatprod.6b00808
95. Kanaoka M, Isogai A et al (1978) Bassianolide, a new insecticidal cyclodepsipeptide from *Beauveria bassiana* and *Verticillium lecanii*. Agric Biol Chem 42:629–635. https://doi.org/10.1080/00021369.1978.10863029
96. Ohyama M, Okada Y et al (2011) Structure-activity relationship of anthelmintic cyclooctadepsipeptides. Biosci Biotechnol Biochem 75(7):1354–1363. https://doi.org/10.1271/bbb.110129
97. Ding G, Chen AJ et al (2012) Sesquiterpenes and cyclopeptides from the endophytic fungus *Trichoderma asperellum* SAMUELS, LIECKF & NIRENBERG. Chem Biodivers 9(6):1205–1212. https://doi.org/10.1002/cbdv.201100185
98. Elsworth JF, Grove JF (1977) Cyclodepsipeptides from *Beauveria bassiana*. Part 1. Beauverolides H and I. J Chem Soc Perkin Trans 13:270–273
99. Elsworth JF, Grove JF (1980) Cyclodepsipeptides from *Beauveria bassiana*. Part 2. Beauverolides A to F and their relationship to isarolide. J Chem Soc Perkin Trans 1 8:1795–1799
100. Grove JF (1980) Cyclodepsipeptides from *Beauveria bassiana*. Part 3. The isolation of beauverolides Ba, Ca, Ja, and Ka. J Chem Soc Perkin Trans 1(12):2878–2880
101. Ding LJ, Yuan W et al (2016) Oryzamides A-E, Cyclodepsipeptides from the sponge-derived fungus *Nigrospora oryzae* PF18. J Nat Prod 79(8):2045–2052. https://doi.org/10.1021/acs.jnatprod.6b00349
102. Vining LC, Taber WA (1962) Isariin, A new depsipeptide from *Isaria cretacea*. Can J Chem 40:1579–1584. https://doi.org/10.1139/v62-239
103. Baute R, Deffieux G et al (1981) New insecticidal cyclodepsipeptides from the fungus *Isaria felina*. I. Production, isolation and insecticidal properties of isariins B, C and D. J Antibiot (Tokyo) 34(10):1261–1265. https://doi.org/10.7164/antibiotics.34.1261
104. Deffieux G, Merlet D et al (1981) New insecticidal cyclodepsipeptides from the fungus *Isaria felina*. II. Structure elucidation of isariins B, C and D. J Antibiot (Tokyo) 34(10):1266–1270. https://doi.org/10.7164/antibiotics.34.1266
105. Langenfeld A, Blond A et al (2011) Insecticidal cyclodepsipeptides from *Beauveria felina*. J Nat Prod 74(4):825–830. https://doi.org/10.1021/np100890n

106. Chiang YM, Szewczyk E et al (2008) Molecular genetic mining of the *Aspergillus* secondary metabolome: discovery of the emericellamide biosynthetic pathway. Chem Biol 15(6): 527–532. https://doi.org/10.1016/j.chembiol.2008.05.010
107. Oh DC, Kauffman CA et al (2007) Induced production of emericellamides A and B from the marine-derived fungus *Emericella* sp. in competing co-culture. J Nat Prod 70(4):515–520. https://doi.org/10.1021/np060381f
108. Kovaleva V, Bukhteeva I et al (2020) Plant defensins from a structural perspective. Int J Mol Sci 21(15):5307. https://doi.org/10.3390/ijms21155307
109. Lehrer RI (2004) Primate defensins. Nat Rev Microbiol 2(9):727–738. https://doi.org/10.1038/nrmicro976
110. Sathoff AE, Samac DA (2019) Antibacterial activity of plant defensins. Mol Plant-Microbe Interact 32(5):507–514. https://doi.org/10.1094/MPMI-08-18-0229-CR
111. Schneider JJ, Unholzer A et al (2005) Human defensins. J Mol Med (Berl) 83(8):587–595. https://doi.org/10.1007/s00109-005-0657-1
112. Koehbach J (2017) Structure-activity relationships of insect defensins. Front Chem 5:45. https://doi.org/10.3389/fchem.2017.00045
113. Qi S, Gao B, Zhu S (2022) A fungal defensin inhibiting bacterial cell-wall biosynthesis with non-hemolysis and serum stability. J Fungi (Basel) 8(2):174. https://doi.org/10.3390/jof8020174
114. Wu J, Gao B, Zhu S (2014) The fungal defensin family enlarged. Pharmaceuticals (Basel) 7(8):866–880. https://doi.org/10.3390/ph7080866
115. Mygind PH, Fischer RL et al (2005) Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus. Nature 437(7061):975–980. https://doi.org/10.1038/nature04051
116. Schneider T, Kruse T et al (2010) Plectasin, a fungal defensin, targets the bacterial cell wall precursor lipid II. Science 328(5982):1168–1172. https://doi.org/10.1126/science.1185723
117. Ostergaard C, Sandvang D et al (2009) High cerebrospinal fluid (CSF) penetration and potent bactericidal activity in CSF of NZ2114, a novel plectasin variant, during experimental pneumococcal meningitis. Antimicrob Agents Chemother 53(4):1581–1585. https://doi.org/10.1128/AAC.01202-08
118. Oeemig JS, Lynggaard C et al (2012) Eurocin, a new fungal defensin: structure, lipid binding, and its mode of action. J Biol Chem 287(50):42361–42372. https://doi.org/10.1074/jbc.M112.382028
119. Zhu S (2008) Discovery of six families of fungal defensin-like peptides provides insights into origin and evolution of the CSαβ defensins. Mol Immunol 45(3):828–838. https://doi.org/10.1016/j.molimm.2007.06.354
120. Zhu S, Gao B, Tytgat J (2005) Phylogenetic distribution, functional epitopes and evolution of the CSαβ superfamily. Cell Mol Life Sci 62(19–20):2257–2269. https://doi.org/10.1007/s00018-005-5200-6
121. Essig A, Hofmann D et al (2014) Copsin, a novel peptide-based fungal antibiotic interfering with the peptidoglycan synthesis. J Biol Chem 289(50):34953–34964. https://doi.org/10.1074/jbc.M114.599878
122. Zhu S, Gao B et al (2012) Dermatophytic defensin with antiinfective potential. Proc Natl Acad Sci U S A 109(22):8495–8500. https://doi.org/10.1073/pnas.1201263109
123. Hallen HE, Luo H et al (2007) Gene family encoding the major toxins of lethal Amanita mushrooms. Proc Natl Acad Sci U S A 104(48):19097–19101. https://doi.org/10.1073/pnas.0707340104
124. Liermann JC, Opatz T et al (2009) Omphalotins E-I, five oxidatively modified nematicidal cyclopeptides from *Omphalotus olearius*. Eur J Org Chem:1256–1262. https://doi.org/10.1002/ejoc.200801068
125. Johnson RD, Lane GA et al (2015) A novel family of cyclic oligopeptides derived from ribosomal peptide synthesis of an in planta-induced gene, gigA, in Epichloë endophytes of grasses. Fungal Genet Biol 85:14–24. https://doi.org/10.1016/j.fgb.2015.10.005

126. Letscher-Bru V, Herbrecht R (2003) Caspofungin: the first representative of a new antifungal class. J Antimicrob Chemother 51(3):513–521. https://doi.org/10.1093/jac/dkg117
127. Johnson MD, Perfect JR (2003) Caspofungin: first approved agent in a new class of antifungals. Expert Opin Pharmacother 4(5):807–823. https://doi.org/10.1517/14656566.4.5.807
128. Stone EA, Fung HB, Kirschenbaum HL (2002) Caspofungin: an echinocandin antifungal agent. Clin Ther 24(3):351–377. https://doi.org/10.1016/s0149-2918(02)85039-1
129. Zhang H, Chen S (2022) Cyclic peptide drugs approved in the last two decades (2001-2021). RSC Chem Biol 3:18–31. https://doi.org/10.1039/d1cb00154j
130. Spampinato C, Leonardi D (2013) Candida infections, causes, targets, and resistance mechanisms: traditional and alternative antifungal agents. Biomed Res Int 2013:204237. https://doi.org/10.1155/2013/204237
131. Sumiyoshi M, Miyazaki T et al (2020) Novel and potent antimicrobial effects of caspofungin on drug-resistant Candida and bacteria. Sci Rep 10(1):17745. https://doi.org/10.1038/s41598-020-74749-8
132. Survase SA, Kagliwal LD et al (2011) Cyclosporin A—a review on fermentative production, downstream processing and pharmacological applications. Biotechnol Adv 29(4):418–435. https://doi.org/10.1016/j.biotechadv.2011.03.004
133. Tugwell P, Pincus T et al (1995) Combination therapy with cyclosporine and methotrexate in severe rheumatoid arthritis. The methotrexate-cyclosporine combination study group. N Engl J Med 333(3):137–141. https://doi.org/10.1056/NEJM199507203330301
134. Yocum DE (1996) Combination therapy with cyclosporin in rheumatoid arthritis. Rheumatology 35(suppl 2):19–23. https://doi.org/10.1093/rheumatology/35.suppl_2.19
135. Galgóczy L, Yap A, Marx F (2019) Cysteine-rich antifungal proteins from filamentous fungi are promising bioactive natural compounds in anti-Candida therapy. Isr J Chem 59:360–370. https://doi.org/10.1002/ijch.201800168
136. Magana M, Pushpanathan M et al (2020) The value of antimicrobial peptides in the age of resistance. Lancet Infect Dis 20(9):e216–e230. https://doi.org/10.1016/S1473-3099(20)30327-3
137. Bills G, Li Y et al (2014) New insights into the echinocandins and other fungal non-ribosomal peptides and peptaibiotics. Nat Prod Rep 31(10):1348–1375. https://doi.org/10.1039/c4np00046c
138. Oide S, Turgeon BG (2020) Natural roles of nonribosomal peptide metabolites in fungi. Mycoscience 61(3):101–110., ISSN 1340-3540. https://doi.org/10.1016/j.myc.2020.03.001

7 Cultivation, Bioactive Metabolites, and Application of Caterpillar Mushroom *Cordyceps militaris*: Current State, Issues, and Perspectives

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Abstract

Cordyceps militaris is a valuable mushroom with wide use in food and medicine. This fungal product usage in many countries, especially in Southeast Asia, has become widespread. There is a growing realization that *C. militaris* can be used as a succedaneum for Chinese cordyceps (*Ophiocordyceps sinensis*) due to their similar chemical characteristics and therapeutic properties. In nature, the complicated life cycle of *C. militaris* consists of teleomorph stage, anamorph stage, and the lifespan of the host insects. The fruiting bodies propagated by inoculation on cereal substrates and silkworm pupae have been successfully mass-produced. A battery of active components such as cordycepin, adenosine, N6-(2-hydroxyethyl)-adenosine, carotenoid, and polysaccharide have been extracted from fruiting body. Evidence shows that *C. militaris* has various bioactivities such as immunomodulatory, anti-inflammatory, antitumor, antimicrobial, insecticidal, anti-fibrotic, liver protection, kidney protection, and pneumonia protection. This fungus finds can be found in functional food, healthcare

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fields, as well as skin care products in East Asian countries represented by China, Japan, and Korea. Full elucidation of the production capacities of different metabolites and the quality control of the products are critically needed in the future. This review will be helpful for the future research and application of this fungus.

Keywords

Cordyceps militaris · Life cycle · Fruiting body cultivation · Cordycepin · Pharmaceutical and therapeutic potential · Industry and application

7.1 Introduction

Caterpillar mushroom, *Cordyceps militaris* (L.) Fr., (Cordycipitaceae, Ascomycetes), is one of the popular edible and medicinal fungi. It is the type species of the modified genus *Cordyceps* of the family Cordycipitaceae [1]. *C. militaris* is more and more regarded as a succedaneum for Chinese cordyceps (*Ophiocordyceps sinensis*) in traditional Chinese medicine on account of similar chemical and medicinal properties [2–4].

There are several resemblances between *C. militaris* and *O. sinensis*; however, they differ in their hosts, appearance, and geographical distribution. The hosts of *C. militaris* are Lepidopteran pupae or larvae, and their fruiting bodies are yellow or orange in color (Fig. 7.1a, b), while the hosts of *O. sinensis* are larvae of *Hepialus*, and the color of their fruiting bodies is dark brown (Fig. 7.1c, d). The natural resource of Chinese cordyceps fungus is scarce, which is only found on the Tibetan Plateau. Being different from the Chinese cordyceps fungus, *C. militaris* is distributed worldwide, including North and South America, Europe, and Asia, but with a low population density in nature.

Cordyceps militaris is well-known because of its radio-protection [5], anti-influenza virus [6], anti-inflammatory, and antitumor [7] activities. A series of components, including cordycepin, adenosine, N6-(2-hydroxyethyl)-adenosine, and carotenoids, have been extracted from the *C. militaris*. The use of *Cordyceps militaris* product in medicinal treatment and health products has been well received in several countries, especially in Southeast Asia. In 2009, the Ministry of Health of the People's Republic of China officially recognized *C. militaris* as a novel food [http://www.moh.gov.cn/publicfiles/business/htmlfiles/zwgkzt/pgg/200903/39591.htm].

Large-scale fermentation and substrate culture have been widely carried out in China to meet the great demand for medicinal and edible products [8, 9]. At present, the fruiting body has been successfully cultivated commercially, which is not only used in medicine and healthcare products but also can be sold directly in supermarkets as edible fungi.

This review will summarize the research progress including the life cycle and fruiting body cultivation, metabolites and pharmacological activities,

Fig. 7.1 Fruiting body of *Cordyceps militaris* and *Ophiocordyceps sinensis* in the wild: (**a**) fruiting body of *C. militaris* out of the ground; (**b**) fruiting body of *C. militaris* and the pupae; (**c**) fruiting body of *O. sinensis* out of the ground; (**d**). fruiting body of *O. sinensis* and its host

pharmaceutical and therapeutic potential, safety and toxicity, as well as industry and applications. The issue of making this prized fungus beneficial to all mankind is critically discussed. This review summarizes the existing research results and lays the foundation for the future research.

7.2 Life Cycle and Fruiting Body Cultivation

7.2.1 Life Cycle of *Cordyceps militaris*

In nature, the complicated life cycle of *C. militaris* comprises of teleomorph stage and anamorph stage as well as the lifespan of the host insects (Fig. 7.2). The hosts are holometabolous insects (Fig. 7.2). The insects could be infected by conidia, ascospores, and hyphae. Under the specific environmental conditions, the ascospores could be ejected and then diffused by wind or water to infect the insects. *C. militaris*

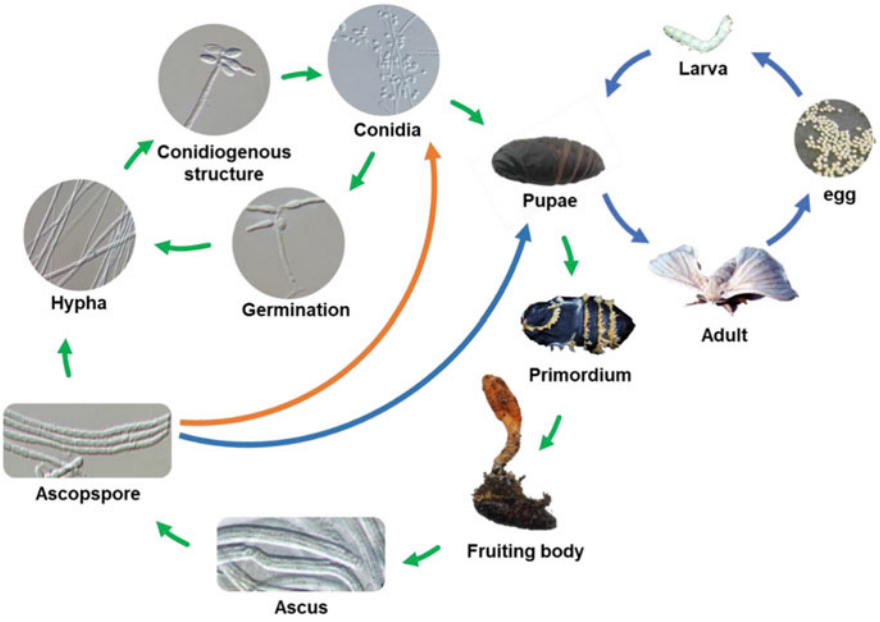

Fig. 7.2 Life cycle of *Cordyceps militaris*

colonizes the living host insects, and when the insects are eventually dead, it switches to necrotrophy. Being different from the *O. sinensis* which is homothallic, *C. militaris* is sexually heterothallic. Interestingly, single mating-type strain could also develop fruiting body without mating and meiosis, but sexual perithecia can't be generated [10].

7.2.2 The Isolates of *Cordyceps militaris*

C. militaris can be isolated from spores and tissues. For spore isolation, ascospores ejected from mature stromata are collected and then spread in sterile Potato Dextrose Agar (PDA) medium. The ascospores can germinate after 2–3 days. For isolation of tissue culture, the fruiting body was sterilized with 70% ethanol, and a piece of tissue was cut and put in sterile PDA medium under aseptic conditions.

7.2.3 Cultivation of Fruiting Bodies of *Cordyceps Militaris* on Cereal Substrates

Fruiting body cultivation on cereal substrates was divided into six stages: spawn production, mycelial growth under dark, coloring after light irradiation, primordium differentiation, fruiting body development, and fruiting body harvest. In a petri plate,

the strain was cultured on Potato Dextrose Agar (PDA), and a sterilizing cutter is used to make five to eight circles with a diameter of 5 mm from the agar plate medium and transfer them to the seed medium, that is, potato dextrose liquid medium. The liquid spawn was cultured in a 500 mL flask with 150 mL of potato dextrose liquid medium in a rotary shaker incubator. Wheat or rice medium was prepared by mixing 30 g of cereal and 45 mL of distilled water in 500 mL glass bottles and was then sterilized. The inoculated cultivation was cultured at 20 °C for 7–10 days under dark condition and then under 12 h light and 12 h dark conditions, with relative humidity greater than 80%. The primordium was induced at temperature of 18–22 °C, high humidity of 70–80% under a light intensity of 400–500 lx. After 60–70 days of culture, fresh fruiting bodies were harvested. The by-products produced in large quantities (mainly culture substrates) are mostly used as animal feed.

7.2.4 Cultivation of Fruiting Bodies of *Cordyceps militaris* on Pupae

We can obtain the complete products similar to the natural products of *C. militaris* by cultivation on pupae. The fruiting bodies of pupae are displayed in Fig. 7.3b; *Bombyx mori* silkworm pupae can be raised for fruiting body cultivation. In order to remove entangled hyphae, the liquid spawn was prepared as mentioned above and then filtered through sterilized gauze. A syringe is used to inject liquid spawn (0.1 mL) into each pupa. The injected pupae were incubated in containers at 20 °C until fungal growth caused their bodies to become stiff and mummified after injection inoculation.

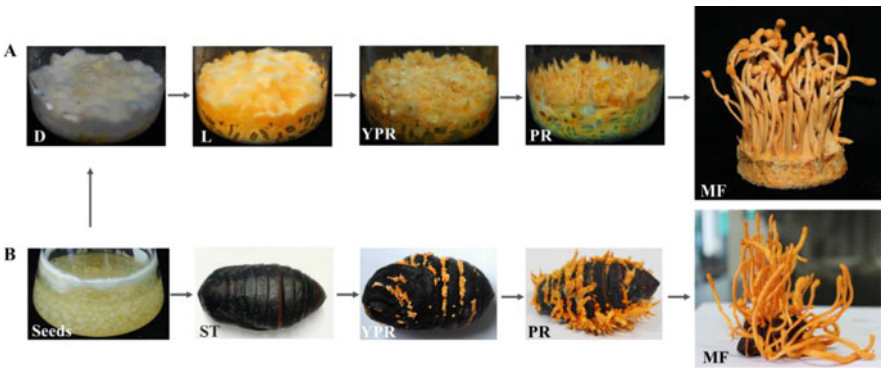

Fig. 7.3 *Cordyceps militaris* fruiting bodies cultivated on wheat medium (**a**) and on pupae (**b**): D (dark); L (light irradiation for 4 d); ST (sclerotium); YPR (young primordium); PR (primordium); MF (mature fruiting body)

7.2.5 Strain Degeneration of *Cordyceps militaris* and Strain Preservation

During the industrial cultivation, the degeneration of *C. militaris* strain frequently results in a decline in growth rate, fruiting body output, and secondary metabolite content, which can cause significant financial losses [11, 12]. The degeneration of *C. militaris* is influenced by environmental factors (such as culture medium, preservation techniques, oxidative stress, and subculture) as well as genetic variation (such as DNA methylation and degeneration-related genes). The primary factor for the degeneration of *C. militaris* strains is genetic variation [13].

Degradation can be avoided or delayed by proper preservation methods. Lyophilization allows *C. militaris* strains to preserve their natural properties and avoid degradation, which is appropriate for long-term preservation (at least 4 years). The characteristics of *C. militaris* strains stored in sterile water are similar to that of −196 °C cryopreservation in liquid nitrogen for at least 1 year. Preservation in sterile water is an effective method for the preservation of *C. militaris* strains, which can be used in factory production. For this fungus, −80 °C cryogenic freezing is not applicable [14].

7.3 Metabolites and Biological Activities

Multiple compounds have been extracted, detected, and purified from *C. militaris*, including cordycepin, cordycepic acid, carotenoids, ergosterol, polysaccharides, nucleosides, and other compounds.

7.3.1 Nucleosides and Analogues

Cordycepin (3′-deoxyadenosine, $C_{10}H_{13}N_5O_3$) is the most important nucleoside analogue which was first purified from *C. militaris* [15, 16], and it has demonstrated a wide range of pharmacological activities. A ribose moiety is attached to a purine (adenine) nucleoside molecule via a β-N9-glycosidic bond in the structure of cordycepin (Fig. 7.4). Most of the ^{14}C-labeled substances (adenosine, adenine, etc.) and ^{3}H -labeled ribose were determined to be potential cordycepin precursors [17, 18]. Therefore, it was assumed that cordycepin was synthesized via purine nucleotide pathway. However, a comprehensive description of cordycepin production was not proposed and confirmed until 2017 [19]. It was demonstrated that adenosine served as the starting point for the sequential reactions of phosphorylation, dephosphorylation, and reduction, which were performed by the Cns1/Cns2 complex (Fig. 7.4).

Various pharmacological activities of cordycepin including antioxidant, anticancer, anti-inflammatory, antidiabetic, and antimicrobial activities and inhibition of platelet aggregation have been reported (Table 7.1). Cordycepin induces both extrinsic apoptotic and caspase-mediated intrinsic regulation [20–23], as well as

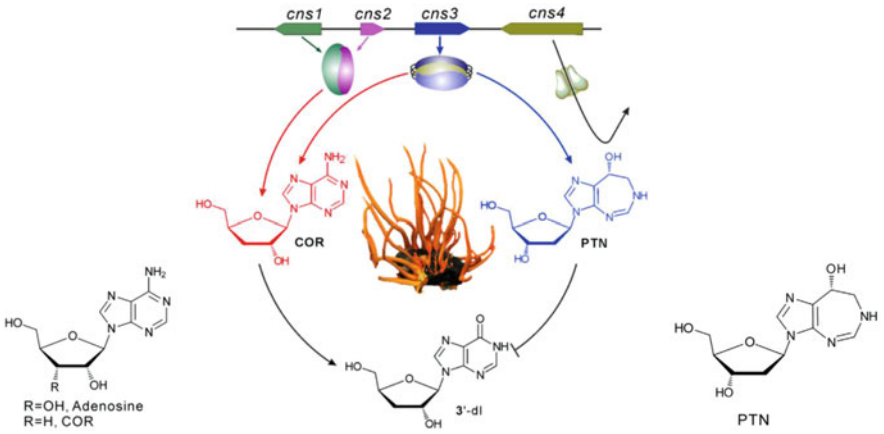

Fig. 7.4 Chemical structures and synthesis pathway of cordycepin and pentostatin [19]: COR, cordycepin; PTN, pentostatin; 3'dI, 3′-deoxyinosine

Table 7.1 Biological activities of metabolites from *Cordyceps militaris*

Metabolites	Biological activities	References
Adenosine	Regulate energy homeostasis, affect the function of various organs and central nervous system activity, and modulate irregular heartbeat, delay recovery of synaptic transmission following spreading depolarization; tumor immunity	[40] [43] [42] [39] [41]
Cordycepin	Anti-inflammatory, antioxidant, antitumor, antidiabetic, antibacterial, platelet aggregation inhibition, hypolipidemic, analgesia, immunomodulatory, antithrombotic effects, and neuroprotective effects	[89] [90] [23] [91] [92] [93] [94] [95] [96]
Cordycepic acid	Anti-lung cancer	[71]
Polysaccharides	Elevating TNF-α and IL-1β secretion in macrophage, increasing nitric oxide production in macrophage, enhancing T lymphocyte proliferation and IL-2 secretion, promoting macrophage phagocytotic and acid phosphatase activities, anti-influenza, stimulating dendritic cell maturation	[97] [98] [99] [100] [101]
Pentostatin	Treatment of several forms of leukemia	[102]
Ergosterol	Anti-inflammatory activity, diuretic activity, anticancer activity, antioxidant, antitumor, inhibition of bladder carcinogenesis promotion	[65] [103] [104] [68]

resistant cancer metastasis [22, 24], and proliferation via MAPK [25, 26], GSK-3β [23, 27], and ERK-JNK pathways [26, 28] found that cordycepin may reduce non-alcoholic fatty liver in ob/ob mice, and the potential mechanism may be mediated by reduced expression of genes involved in inflammation and lipid formation. Cordycepin could also modulate the central nervous system by suppressing excitatory synaptic transmission via a presynaptic mechanism in rat hippocampal slices [29]. Besides, cordycepin could shield dopamine neurons from rotenone-induced apoptosis by effectively improving mitochondrial dysfunction [30]. Cordycepin could stimulate Wnt/β-catenin signaling pathway through the activation of adenosine receptors, which may accelerate the process of tissue remodeling, showing its potential in the treatment of skin wounds. In brief, cordycepin showed important therapeutic properties in various ailments, as well as nutraceuticals for chronic disease prevention [31].

Another adenosine analogue, pentostatin (2′-deoxycoformycin, $C_{11}H_{16}N_4O_4$), was first isolated from *Streptomyces antibioticus* in the 1970s [32], which is an adenosine deaminase irreversible inhibitor preventing the deamination of cordycepin to 3′-deoxyinosine (Fig. 7.4). It was confirmed that an individual gene cluster can generate both cordycepin and pentostatin [19]. Pentostatin helps to suppress deamination of cordycepin to 3′-deoxyinosine in vivo, probably by blocking adenosine deaminase in *C. militaris*.

These two adenosine analogues produced in tandem follow a bacterial-like protector-protégé strategy of purine metabolism. Pentostatin, in combination with cordycepin, has been reported in clinical trials of refractory TdT-positive leukemia [http://clinicaltrials.gov/show/NCT00709215].

Pentostatin, a purine analogue, was authorized for use in the United States in 1991 to treat hairy cell leukemia and T cell lymphomas [33]. Pentostatin, as a strong adenosine deaminase (ADA) inhibitor, has also been investigated for use in preventing GVHD (graft-versus-host disease) [34–36]. The combination of cordycepin and pentostatin showed reduction of epimastigotes and trypomastigotes in vitro, but no therapeutic effect infected by *Trypanosoma cruzi* in mice [37]. Pentostatin could also have common adverse effects, including fever, headache, nausea, fatigue, infection, anorexia, vomiting, and rash [33].

Additional nucleosides were isolated from *C. militaris* such as adenine, adenosine, uracil, uridine, guanidine, guanosine, hypoxanthine, and N6-(2-Hydroxyethyl)-adenosine.

Adenosine ($C_{10}H_{13}N_5O_4$, Fig. 7.5) is involved in various physiological and pathophysiological regulatory mechanisms [38] and has been used as quality standard of some cordyceps products. Adenosine serves as a signaling substance in addition to being a significant component of ribonucleic acids and adenine nucleotides. It can regulate energy homeostasis, affect the function of various organs and central nervous system activity, and modulate irregular heartbeat (Table 7.1). Extracellular adenosine acts as a signaling molecule by binding to adenosine receptors, which could trigger multiple signaling pathways to regulate energy homeostasis and affect the operation of many organs [39]. Adenosine can be used for a variety of important therapies, including regulating central nervous system

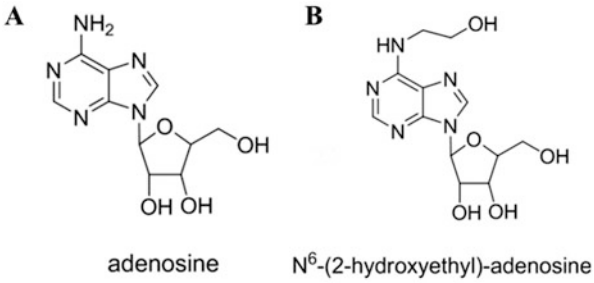

Fig. 7.5 Chemical structures of adenosine (**a**) and N6-(2-hydroxyethyl)-adenosine (**b**) [46]

(CNS) function and modulating irregular heartbeat (arrhythmias) [40, 41]. The accumulation of extracellular adenosine in neural tissue resulted in a delay in the synaptic recovery process after spreading depolarization [42]. Leone and Emens described the function of adenosine signaling in regulating tumor immunity, emphasizing underlying therapeutic targets in the pathway [43]. Besides, behavioral and physiological symptoms produced by insufficient adenosine were consistent with many comorbidities of autism in rodents [44], and adenosine might be a novel therapeutic target for autism in rodents and humans [45].

The first calcium antagonist of biological origin, N6-(2-hydroxyethyl)-adenosine ($C_{12}H_{17}N_5O_5$, HEA, Fig. 7.5), is also an inotropic agent [46]. Among the over 500 species of *Cordyceps* sensu lato, HEA could be produce by only a few species such as *C. pruinosa*, *C. militaris*, and *I. tenuipes* [47]. Recent studies have shown that HEA has a variety of biological functions. In pharmacological studies, cerebral and coronary circulation could be regulated by HEA which acts as a sedative [48]. It can prevent K562 erythroleukemia cells from proliferating in vitro [49]. The analgesic mechanism of HEA differs from that of opioids, a widely used analgesic drug. More importantly, the benefit of HEA over opioids is that it is neither addictive nor pepsin-affected [50]. Peng et al. reported that HEA shielded mice from renal ischemia reperfusion damage [51]. Additionally, HEA reduced the pro-inflammatory reactions brought on by lipopolysaccharide through inhibiting the toll-like receptor 4-mediated nuclear factor-κB signalling pathway [52]. A recent study showed that HEA targeting an adenosine receptor showed insecticidal activity to *Plutella xylostella* [53], suggesting an environmentally friendly pesticide [54].

7.3.2 Polysaccharide

One of the major biologically active elements of *C. militaris* is polysaccharide. The healthcare and pharmacological activities of *C. militaris* were confirmed by a mass of animal experiments and clinical experiments (Table 7.1). *Cordyceps militaris* contains various polysaccharides, which mainly composed of mannose, glucose, and galactose in varying molar ratios [55–58]. In addition, several isolated polysaccharides contained monosaccharides such as xylose, rhamnose, and arabinose [59, 60]. The change of monosaccharide molar ratio of polysaccharides may be

related to raw materials, separation and purification techniques, etc. Polysaccharides had antiviral, antioxidative, anti-inflammatory, antitumor, neuroprotective, antihypertensive, and immunomodulatory biological effects [6, 61, 62]. The biological functions and healthcare functions of polysaccharides were reviewed by Zhang et al. [63].

7.3.3 Ergosterol

Ergosterol ($C_{28}H_{44}O$, Fig. 7.6) is an important sterol component of *C. militaris* which is an indispensable source of fat-soluble vitamin D and can be determined by HPLC method [64]. Ergosterol was first isolated from *Claviceps purpurea* (Fr.) Tul in 1898; subsequently, it was discovered in a variety of edible and medicinal fungi. As an important component of fungal cell membranes, it plays a significant role in ensuring cell viability, membrane integrity, and completion of the cell cycle.

Ergosterol safeguards the phospholipids in cell membranes from oxidation by operating as an antioxidant in a manner akin to vitamin E [65]. Ergosterol significantly improved the anticancer effect [66]. By regulating the TGF-β1/Smad2 signaling pathway, ergosterol decreased the proliferation of mesangial cells and the subsequent deposition of ECM [67]. Both oral administration and intraperitoneal administration of ergosterol inhibited the promotion of bladder carcinogenesis with saccharin sodium, and the last one showed a stronger effect [68].

7.3.4 Cordycepic Acid

Cordycepic acid ($C_6H_{14}O_6$) also known as D-mannitol was first determined from *O. sinensis* which was reported to be a quinic acid isomer [69]. As a tetrahydroxy monobasic acid, it forms a crystalline tetra-acetate, a mono-methyl ester, and a tetrabenzoate in *O. sinensis* [69]. The structure and chemical composition of mannitol between *O. sinensis* and *C. militaris* are substantially uniform. Cordycepic acid can

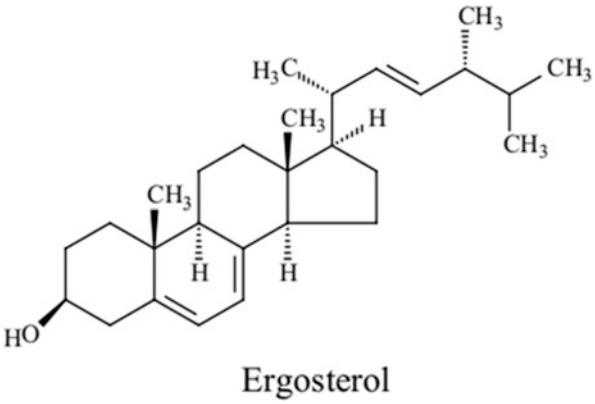

Fig. 7.6 Chemical structure of ergosterol [64]

improve LPS-induced hepatic stem cell inflammation phenotype and TGFB1-induced hepatic stem cell fibrosis response [70]. By regulating Nrf-2/HO-1/NLRP3/NF-κB signal in nude mice, cordycepic acid has a significant antitumor effect on carcinoma of the lung [71].

7.3.5 Carotenoids

Carotenoids are a class of pigmented chemicals produced by plants and microbes but not by animals [72]. All carotenoids possess a polyisoprenoid structure [73]. Under the illumination condition, the colony of *C. militaris* exhibited the color of orange with high carotenoid content in mycelia [74]. Carotenoids isolated from the fruiting body of *C. militaris* include lutein, zeaxanthin, and four cordyxanthins (Fig. 7.7) [75, 76]. *C. militaris* is rich in carotenoids, with a greater amount than other known mushrooms. It was proposed that carotenoid concentration should be used as a quality criteria for commercial products of this valuable fungus [77].

As a family of natural organic pigment compounds, carotenoid could decrease oxidative stress, which is related to the progression [78], and the progression of age-related diseases [79]. Cell-mediated immune function and sexual attraction changed with the carotenoid content of the diet in male zebra finches [80]. Carotenoid had also characteristic functions such as cell cycle inhibition, improvement of gap-junctional communication, and induction of cell differentiation and apoptosis [81].

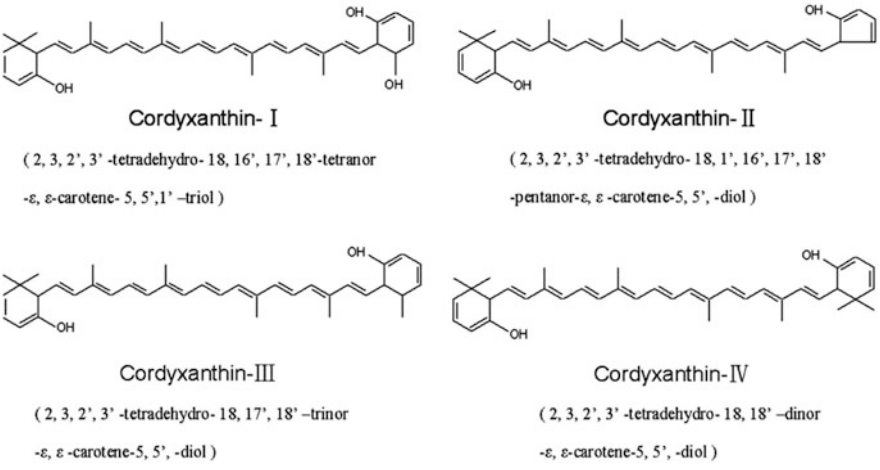

Fig. 7.7 Chemical structure of four cordyxanthins [75]

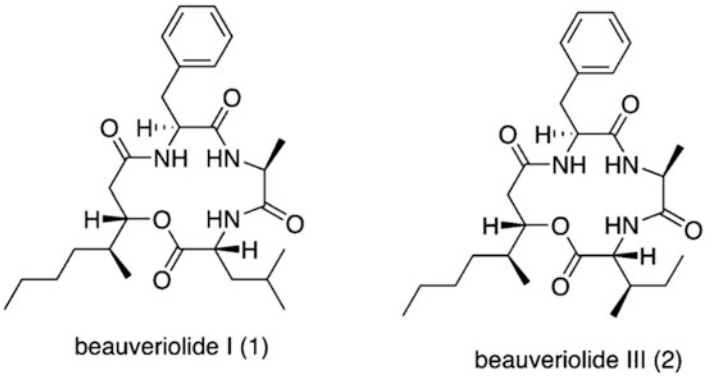

Fig. 7.8 Chemical structure of beauveriolides [88]

7.3.6 Other Compounds

5,5'-Dibuthoxy-2,2′-bifuran is initially found from plants. It was extracted for the first time from *C. militaris* that showed antibacterial activity against *Bacillus subtilis* and *Escherichia coli* [82].

In addition to the above compounds, superoxide dismutase (SOD) is an active protease that can eliminate oxidative stress to protect cells from damage of aerobic metabolite [83]. Cephalosporolides C, E, and F, ten-membered macrolides ($C_{10}H_{14}O_4$), 2-carboxymethyl-4-(3′-hydroxybutyl) furan, and pyridine-2, 6-dicarboxylic acid ($C_7H_5NO_4$) were also extracted from *C. militaris* [84]. *C. militaris* lectin showed no hemagglutination activity in human ABO erythrocytes but had agglutination activity in mouse and rat erythrocytes [85]. Cerebrosides with anti-inflammatory and anti-PTP1B activities were isolated in *C. militaris* [86, 87]. Recently, *C. militaris* genome mining revealed a possible gene cluster for acyl-CoA: cholesterol acyltransferase inhibitor beauveriolides (Fig. 7.8) [88].

Apart from the metabolites described above, genomic analysis of *C. militaris* identified secondary metabolite clusters of seven polyketide synthases (PKS), five NRPS-PKS, eight non-ribosomal peptide synthetases (NRPS), and four terpene synthases (TS). Many more metabolites should be expected to be dug out.

7.4 Pharmaceutical and Therapeutic Potential of *Cordyceps militaris*

Cordyceps has long been used as a tonifying kidney and lung health medicine, as well as to treat asthma, tuberculosis, chronic bronchitis, and other respiratory illnesses. Evidence showed that *C. militaris* are beneficial to act as anti-angiogenic, anti-inflammatory, antitumor, antioxidant, anti-aging, immunomodulatory, antimicrobial, antiviral, anti-protozoal, insecticidal, anti-fibrotic, hypolipidemic, liver

Table 7.2 List of pharmacological effects of *Cordyceps militaris*

Pharmacological effects	Samples of *Cordyceps militaris*	References
Anti-angiogenic	*C. militaris* extract	[105]
Antitumor/anti-proliferatory	*C. militaris* protein	[106, 107]
	Aqueous extract	
	BuOH extracts	[108]
	Crude polysaccharide	[109]
	Alcohol extract (anti-non-small cell lung cancer)	[110]
Induce apoptosis	Aqueous extract	[111]
		[106, 107]
	Alcohol extract	[110]
Immunomodulatory	Purified cordycepin	
	Homogeneous polysaccharide (CMP-III)	[112]
Anti-inflammatory	Water extract	[110]
	Constituents isolated from *C. militaris*	[113]
Hypoglycemic	*C. militaris* extract	[114]
	Exopolysaccharide	[109]
Anti-fibrotic	EPC from *C. militaris*	[115]
Improves chronic kidney disease	Cordycepin	[87]
Anti-PTP1B (protein tyrosine phosphatase 1B)	A new cerebroside	[116]
Antioxidant	*C. militaris* extracts-mediated nanoemulsion	[117]
Against liver dysfunction and obesity	Fermented *C. militaris* extract	[118]
Attenuated doxorubicin-induced cytotoxic effects in chemotherapy	Polysaccharides	[119]
Against bisphenol A-induced reproductive damage	*C. militaris* extract	[23]
Inhibit RANKL-induced osteoclast differentiation	*C. militaris* mushroom and cordycepin	[120]
Lipid-lowering	Two novel polysaccharides, CM1 and CMS	[121]
Prevent Pb^{2+}-induced liver and kidney toxicity	Extracellular polysaccharide	[122]
Alleviated allergic rhinitis	Ethanol extract prepared from silkworm pupa-cultivated *C. militaris* fruiting bodies	[123]
Anti-atherosclerotic	Alkaline-extracted polysaccharides were obtained from the fruiting body	[124]
Antidepressant	Water extract	[125]

protection, and lung protection. The pharmaceutical and therapeutic potential of *C. militaris* and the samples used were listed in Table 7.2.

7.5 Safety and Toxicity

Mycotoxin contamination in food and feed has long posed a threat to human and animal health [126]. *C. militaris* has been consumed for many years, and its safety has also been demonstrated because the genome lacks genes known to be responsible for human mycotoxins [127]. The sub-chronic toxicity experiments of submerged mycelial of *C. militaris* in rats for 90 days showed that the threshold of no adverse reaction of *C. militaris* hyphae to male and female SD rats was 4000 mg per kg BW per day [128]. *C. militaris* extract has a good effect on mild liver dysfunction by inhibiting lipid accumulation and slowing down the progression of fatty liver [129]. It was proved that cordycepin is a non-toxic and non-mutagenic compound by test and in rat model. The hematological, blood chemistry, and tissue microanatomical characteristics of rats fed cordycepin daily for 30 consecutive days were not significantly different from those of normal rats [130].

However, the production of highly effective cordycepin and penstatin is a key criterion for the selection of strains for mass production. Similar to the side effects of pentostatin and adenosine analogues, there have been anecdotal reports of the adverse effects of nausea and diarrhea after ingestion of products with high levels of cordycepin/pentostatin [66].

7.6 Industry and Application

In traditional Chinese medicine, *Cordyceps* fungi have been employed for centuries. For various biological and pharmacological activities of *C. militaris*, there are many applications in functional food, healthcare fields, as well as skin care product in China, Japan, Korea, and other East Asian countries. Mass production of *C. militaris* inoculated on cereal substrates and silkworm pupae has long been successful. Currently, cordyceps have turned to be a big industry. Without taking into account the yield of *C. militaris* in South Korea, Japan, Vietnam, and Thailand, yearly output amounts to 90, 559 tons in 2018 and annual value of production was about 10 billion RMB in China.

Fruiting bodies have been commercially cultivated on a large scale, not only for medicine and healthcare products but also for direct consumption in supermarkets as edible fungi. There are a great number of products of dry or fresh fruiting bodies of *C. militaris* on the market (Fig. 7.9). The famous cordyceps recipe is made with duck. *C. militaris* chicken soup is also popular (Fig. 7.10).

In 2009, the Ministry of Health of the People's Republic of China officially recognized *C. militaris* as a novel food. The fruiting bodies of *C. militaris* have also been processed into a variety of different products, including cordyceps noodle, drink, tea, and so on (Fig. 7.11). In addition, 36 officially healthy foods are made from *C. militaris*. Most of them were used for improving the immunity. *C. militaris* is used in cosmetic, and there are products of mask, lotion, moisturizer, and so on (Fig. 7.12). In recent years, it has been reported that the solid-based residues (SBRs) of *Cordyceps* mushroom fruiting body can be used as a potential source of crude

Fig. 7.9 Fresh and dried fruiting bodies of *Cordyceps militaris* in the market. (**a**) products of fresh fruiting body; (**b**) products of dry fruiting body

Fig. 7.10 Dishes cooking with *Cordyceps militaris*

Fig. 7.11 Processed foods of *Cordyceps militaris*. (**a**) noodle; (**b**) beverage; (**c**) tea; (**d**) health food; (**e**) candy; (**f**) health products with high cordycepin content

Fig. 7.12 Cosmetics of *Cordyceps militaris*

extracts for cosmetics and can be further used as multifunctional ingredients in cosmetics and cosmeceuticals [131]. Nowadays, an enormous industry building on *C. militaris* has been flourished in China.

C. militaris mycelia powder (Z20030034) and capsule (Z20030035) (Fig. 7.13) which are manufactured by Jilin Zhong sheng Pharmaceutical Co., Ltd. have been approved by the Ministry of Health of the People's Republic of China. The pharmacological effects of the medicines are shown in the drug instruction.

Fig. 7.13 Capsule (Z20030035) manufactured by Jilin Zhong sheng Pharmaceutical Co., Ltd., China

7.7 Perspective

7.7.1 *Cordyceps militaris* and the Other Species of *Cordyceps* s.l.

Cordyceps s.l. include three families of Hypocrealean fungi, i.e., Cordycipitaceae, Ophiocordycipitaceae, and Clavicipitaceae. Only a few fungi of cordyceps have been employed in traditional medicine, such as *Ophiocordyceps sinensis*, *C. militaris*, and *C. cicadae* (syn. *Isaria cicadae*). Among them, *C. militaris*, *C. cicadae*, and *O. guangdongensis* have been approved as novel foods by the Ministry of Health of the People's Republic of China. All the related fungi were called as *Cordyceps* fungi. Here, we called on that the accurate species name should be used both in the scientific research and industrial production in case of confusion.

7.7.2 In-Depth Investigations of the Secondary Metabolites of *Cordyceps militaris*

Except cordycepin, the polysaccharides of other pharmacologically active compounds need to be identified and elucidated in relation to their structure and function. In contrast to the few known metabolites, *C. militaris* genome encodes a series of biosynthetic gene clusters with the potential to generate a large number of unknown compounds. Full elucidation of the production capacities of different secondary metabolites is an urgent need in the future.

7.7.3 Quality Control for *Cordyceps militaris* Products

Although *C. militaris* has become a massive industry and various products have been in market, there are no special active components for the products except the common ones such as polysaccharides and adenosine. One of the major problems limiting development in modern industry is the lack of quality control over medicinal fungi and other traditional Chinese medicines. Chemical (secondary metabolites) and protein fingerprinting should be the choice for the quality control in industry.

7.8 Conclusions

Cordyceps militaris has attracted considerable research and commercial interest because it contains active compounds beneficial to human health, and the fruiting bodies are relatively easy to grow with a short growth period. We believe the fruiting body and the products of *C. militaris* will benefit mankind more and more in the future.

References

1. Sung G, Hywel-Jones NL, Sung JM, Luangsa-ard JJ, Shrestha B et al (2007) Phylogenetic classification of *Cordyceps* and the *Clavicipitaceous* fungi. Stud Mycol 57:5–59
2. Dong JZ, Lei C, Ai XR, Wang Y (2012) Selenium enrichment on *Cordyceps militaris* link and analysis on its main active components. Appl Biochem Biotechnol 166:1215–1224
3. Gao SY, Wang FZ (2008) Research of commercialized cultivation technology on *cordyceps militaris*. North Hortic 9:212–215. (in Chinese)
4. Yue GGL, Lau CBS, Fung KP, Leung PC, Ko WH (2008) Effects of Cordyceps sinensis, *Cordyceps militaris* and their isolated compounds on ion transport in Calu-3 human airway epithelial cells. J Ethnopharmacol 117:92–101
5. Jeong MH, Park YS, Jeong DH, Lee CG, Kim JS et al (2014) In vitro evaluation of *Cordyceps militaris* as a potential radioprotective agent. Int J Mol Med 34:1349–1357
6. Lee HH, Park H, Sung GH, Lee K, Lee T et al (2014) Anti-influenza effect of *Cordyceps militaris* through immunomodulation in a DBA/2 mouse model. Mikrobiol Zh 52:696–701
7. Liu F, Zhu ZY, Sun XL, Gao H, Zhang YM (2017a) The preparation of three selenium-containing *Cordyceps militaris* polysaccharides: characterization and anti-tumor activities. Int J Biol Macromol 99:196–204
8. Dai JJ, Fan T, Wu CH, Xiao LZ, Tian SF (2007) Summarization of the study on the artificial cultivation of *Cordyceps militaris* link. J Anhui Agric Sci 35:5469–5471. (in Chinese)
9. Gu YX, Wang ZS, Li SX, Yuan QS (2007) Effect of multiple factors on accumulation of nucleosides and bases in *Cordyceps militaris*. Food Chem 102:1304–1309
10. Lu YZ, Xia YL, Luo FF, Dong CH, Wang CS (2016) Functional convergence and divergence of mating-type genes fulfilling in *Cordyceps militaris*. Fungal Genet Biol 2016(88):35–43
11. Xiong CH, Xia YL, Zheng P, Wang CS (2013) Increasing oxidative stress tolerance and subculturing stability of *cordyceps militaris* by overexpression of a glutathione peroxidase gene. Appl Microbiol Biotechnol 97:2009–2015
12. Yin J, Xin XD, Weng YJ, Gui ZZ (2017) Transcriptome-wide analysis reveals the progress of *cordyceps militaris* subculture degeneration. PLoS One 12:e0186279
13. Lou HW, Lin JF, Guo LQ, Wang XW, Tian SQ et al (2019) Advances in research on *Cordyceps militaris* degeneration. Appl Microbiol Biotechnol 103:7835–7841
14. Liu Q, Wang F, Liu KB, Dong CH (2018) Influence of strain preservation methods on fruiting body growth and metabolite production by the medicinal mushroom *Cordyceps militaris* (ascomycetes). Int J Med Mushrooms 20:1003–1011
15. Ahn YJ, Park SJ, Lee SG, Shin SC, Choi DH (2000) Cordycepin: selective growth inhibitor derived from liquid culture of *Cordyceps militaris* against *Clostridium* spp. J Agric Food Chem 48:2744–2748
16. Cunningham KG, Manson W, Spring FS, Hutchinson SA (1950) Cordycepin, a metabolic product isolated from cultures of *Cordyceps militaris* (Linn.) link. Nature 166:949–949
17. Kredich NM, Guarino AJ (1961) Homocitrullylaminoadenosine, a nucleoside isolated form *Cordyceps militaris*. J Biol Chem 236:3300–3302

18. Lennon MB, Suhadolnik RJ (1976) Biosynthesis of 3'-Deoxydenosine by cordyceps-militaris-mechanism of reduction. Biochim Biophys Acta-Biomembr 425:532–536
19. Xia YL, Luo FF, Shang YF, Chen PL, Lu YZ, Wang CS (2017) Fungal cordycepin biosynthesis is coupled with the production of the safeguard molecule pentostatin. Cell Chem Biol 24:1479–1489
20. Shao LW, Huang LH, Yan S, Jin JD, Ren SY (2016) Cordycepin induces apoptosis in human liver cancer HepG2 cells through extrinsic and intrinsic signaling pathways. Oncol Lett 12: 995–1000
21. Tian T, Song L, Zheng Q, Hu XJ, Yu RM (2014) Induction of apoptosis by *Cordyceps militaris* fraction in human chronic myeloid leukemia K562 cells involved with mitochondrial dysfunction. Pharmacogn Mag 10:325–331
22. Tao XD, Ning Y, Zhao XW, Pan T (2016) The effects of cordycepin on the cell proliferation, migration and apoptosis in human lung cancer cell lines A549 and NCI-H460. J Pharm Pharmacol 68:901–911
23. Wang J, Chen C, Jiang ZH, Wang M, Jiang H, Zhang XY (2016) Protective effect of *Cordyceps militaris* extract against bisphenol an induced reproductive damage. Syst Biol Reprod Med 62:249–257
24. Robinson DR, Wu YM, Lonigro RJ, Vats P, Cobain E et al (2017) Integrative clinical genomics of metastatic cancer. Nature 548:297–303
25. Baik JS, Mun SW, Kim KS, Park SJ, Yoon HK et al (2016) Apoptotic effects of Cordycepin through the extrinsic pathway and p38 MAPK activation in human glioblastoma U87MG cells. J Microbiol Biotechnol 26:309–314
26. Hwang JH, Joo JC, Kim DJ, Jo E, Jang IS (2016) Cordycepin promotes apoptosis by modulating the ERK-JNK signaling pathway via DUSP5 in renal cancer cells. Am J Cancer Res 6:1758–1771
27. Fishman P, Bar-Yehuda S, Liang BT, Jacobson KA (2012) Pharmacological and therapeutic effects of A3 adenosine receptor agonists. Drug Discov 17:359–366
28. Zhong L, Zhao L, Yang F, Yang W, Sun Y et al (2017) Evaluation of anti-fatigue property of the extruded product of cereal grains mixed with *Cordyceps militaris* on mice. J Int Soc Sport Nutr 14:15
29. Yao LH, Huang JN, Li CH, Li HH, Yan WW et al (2013) Cordycepin suppresses excitatory synaptic transmission in rat hippocampal slices via a presynaptic mechanism. CNS Neurosci Ther 19:216–221
30. Jiang X, Tang PC, Chen Q, Zhang X, Fan YY et al (2019) Cordycepin exerts neuroprotective effects via an anti-apoptotic mechanism based on the mitochondrial pathway in a rotenone-induced parkinsonism rat model. CNS Neurol Disord Drug Targets 18:609–620
31. Devi A, Chennakesavulu S, Suresh C, Reddy ABM (2018) In: Rani V, Yadav UCS (eds) Nutraceuticals and their role in human health and disease. Functional food and human health. Springer Nature, Singapore, pp 375–403
32. Woo PWK, Dion HW, Lange SM, Dahl LF & Durham LJ (1975) Cheminform abstract: a novel adenosine and Ara-A, deaminase inhibitor, (R)-3-(2-deoxy-beta-D-erythropento-furanosyl)-3, 6, 7, 8-tetrahydroimidazo (4, 5-D) (1, 3) diazepin-8-OL. Chem. Inform. 6: Abstract 446
33. Lamanna N, Kay NE (2009) Pentostatin treatment combinations in chronic lymphocytic leukemia. Clin Adv Hematol Oncol 7:386–392
34. Bolanos MJ, Jacobsohn DA, Margolis J, Ogden A, Wientjes MG et al (2005) Pentostatin in steroid-refractory acute graft-versus-host disease. J Clin Oncol 23:2661–2668
35. Jacobsohn DA, Chen AR, Zahurak M, Piantadosi S, Anders V, Bolanos MJ (2007) Phase II study of Pentostatin in patients with corticosteroid-refractory chronic graft-versus-host disease. J Clin Oncol 25:4255–4261
36. Parmar S, Andersson BS, Couriel D, Munsell MF, Fernandez-Vina M et al (2011) Prophylaxis of graft-versus-host disease in unrelated donor transplantation with pentostatin, tacrolimus,

and mini-methotrexate: a phase I/II controlled, adaptively randomized study. J Clin Oncol 29: 294–302
37. do Carmo GM, de Sa Ma F, Grando TH, Gressler LT, Baldissera MD et al (2019) Cordycepin (3′-deoxyadenosine) and pentostatin (deoxycoformycin) against *Trypanosoma cruzi*. Exp Parasitol 199:47–51
38. Jacobson KA, Hoffmann C, Cattabeni F, Abbracchio MP (1999) Adenosine-induced cell death: evidence for receptor-mediated signalling. Apoptosis 4:197–211
39. Novotný J (2015) Adenosine and its role in physiology. Cesk Fysiol 64:35–44
40. Choudhury H, Chellappan DK, Sengupta P, Pandey M, Gorain B (2019) Adenosine receptors in modulation of central nervous system disorders. Curr Pharm Des 25:2808–2827
41. Nattel S (2012) Adenosine and atrial arrhythmias: exploring electrophysiological mechanisms in vivo. PACE-Pacing Clin Electrophysiol 35:553–555
42. Lindquist BE, Shuttleworth CW (2017) Evidence that adenosine contributes to Leao's spreading depression in vivo. J Cereb Blood Flow Metab 37:1656–1669
43. Leone RD, Emens LA (2018) Targeting adenosine for cancer immunotherapy. J Immunother Cancer 6:57
44. Mcpartland J, Volkmar FR (2012) Autism and related disorders. Handb Clin Neurol 106:407
45. Masino SA, Kawamura M Jr, Cote JL, Williams RB, Ruskin DN (2013) Adenosine and autism: a spectrum of opportunities. Neuropharmacology 68:116–121
46. Furuya T, Hirotani M, Matsuzawa M (1983) N6-(2-hydroxyethyl) adenosine, a biologically active compound from cultured mycelia of *Cordyceps* and *Isaria* species. Phytochemistry 22: 2509–2512
47. Liu K, Wang F, Wang WZ, Dong CH (2017b) *Beauveria bassiana*: a new N6-(2-hydroxyethyl)-adenosine-producing fungus. Mycology 8:259–266
48. Wang DM, Liu XH, Guo H, Huang JH, Wang L (2013) Design, synthesis and biological activity evaluation of adenosine analogues. Acta Pharm Sin 48:881–886. (in Chinese)
49. Zhu LN, Xue JJ, Liu YF, Zhou S, Tang QJ (2013) Isolation, purification and anti-tumor activity of N6-(2-hydroxyethyl)-adenosine from the fruiting body *Cordyceps militaris* cultured. Acta Edulis Fungi 20:62–65. (in Chinese)
50. Chai YQ, Wei ZM, Chen ZA, Li XL, Liu YG et al., (2004) N6-(2-hydroxyethyl)-adenosine' application in the preparation of analgesic drugs. China, ZL200410094511.0 (in Chinese)
51. Peng XX, Chai YQ, Zhu BC, Jin YW, Li XL et al (2015) The protective effects of N~6-(2-hydroxyethyl)-adenosine extracted from *Ophiocordyceps sobolifera* on renal ischemia reperfusion injury (IRI) in mice. Mycosystema 34:311–320
52. Lu MY, Chen CC, Lee LY, Lin TW, Kuo CF et al (2015) N6-(2-hydroxyethyl) adenosine in the medicinal mushroom *Cordyceps cicadae* attenuates lipopolysaccharide-stimulated pro-inflammatory responses by suppressing TLR4-mediated NF-κB signaling pathways. J Nat Prod 78:2452–2460
53. Fang M, Chai YQ, Chen GV, Wang HD, Huang B (2016) N-6-(2-hydroxyethyl)-adenosine exhibits insecticidal activity against *Plutella xylostella* via adenosine receptors. PLoS One 11: e0162859
54. Chai YQ, Chen GJ, Jin YW, Liu YG, Li XL et al., (2015) Use of N6-(2-hydroxyethyl)-adenosine in preparation of crop pesticide. USA, US20140256669 A1
55. Lee JS, Kwon JS, Won DP, Lee KE, Shin WC et al (2010a) Study on macrophage activation and structural characteristics of purified polysaccharide from the liquid culture broth of *Cordyceps militaris*. Carbohydr Polym 82:982–988
56. Lee JS, Kwon JS, Yun JS, Pahk JW, Shin WC et al (2010b) Structural characterization of immunostimulating polysaccharide from cultured mycelia of *Cordyceps militaris*. Carbohydr Polym 80:1011–1017
57. Smiderle FR, Sassaki GL, Griensven LV, Iacomini M (2013) Isolation and chemical characterization of a glucogalactomannan of the medicinal mushroom Cordyceps militaris. Carbohydr Polym 97:74–80

58. Yu RM, Yang W, Song LY, Yan CY, Zhang Z et al (2007) Structural characterization and antioxidant activity of a polysaccharide from the fruiting bodies of cultured *Cordyceps militaris*. Carbohydr Polym 70:430–436
59. Chen R, Jin CG, Li HP, Liu ZQ, Lu J et al (2014) Ultrahigh pressure extraction of polysaccharides from *Cordyceps militaris* and evaluation of antioxidant activity. Sep Purif Technol 134:90–99
60. Dong CH, Guo SP, Wang WF, Liu XZ (2015) Cordyceps industry in China. Mycology 6:121–129
61. Rodrigues C, Sousa C, Lopes JA, Novais A, Peixe L (2020) A front line on klebsiella pneumoniae capsular polysaccharide knowledge: fourier transform infrared spectroscopy as an accurate and fast typing tool. Msystems 5:e00386–e00319
62. Sims IM, Frese SA, Walter J, Loach D, Wilson M et al (2011) Structure and functions of exopolysaccharide produced by gut commensal *Lactobacillus reuteri* 100-23. ISME J 5:1115–1124
63. Zhang J, Wen C, Gu J, Ji C, Duan Y et al (2019) Effects of subcritical water extraction microenvironment on the structure and biological activities of polysaccharides from *Lentinus edodes*. Int J Biol Macromol 123:1002–1011
64. Li SP, Li P, Lai CM, Gong YX, Kan KKW et al (2004) Simultaneous determination of ergosterol, nucleosides and their bases from natural and cultured *Cordyceps* by pressurized liquid extraction and high-performance liquid chromatography. J Chromatogr A 1036:239–243
65. Dupont S, Lemetais G, Ferreira T, Cayot P, Gervais P et al (2012) Ergosterol biosynthesis: a fungal pathway for life on land? Evolution 66:2961–2968
66. Chen YJ, Wu YQ, Li SL, Du SM, Hao XM et al (2021) Large-scale isolation and antitumor mechanism evaluation of compounds from the traditional Chinese medicine *Cordyceps militaris*. Eur J Med Chem 212:113142
67. Dong ZH, Sun YY, Wei GG, Li SY, Zhao ZX (2019) Ergosterol ameliorates diabetic nephropathy by attenuating mesangial cell proliferation and extracellular matrix deposition via the TGF-β1/Smad2 signaling pathway. Nutrients 11:483–500
68. Yazawa Y, Ikarashi N, Hoshino M, Kikkawa H, Sakuma F (2020) Inhibitory effect of ergosterol on bladder carcinogenesis is due to androgen signaling inhibition by brassicasterol, a metabolite of ergosterol. J Nat Med 74:680–688
69. Chatterjee R, Srinivasan KS, Maiti PC (1957) *Cordyceps sinensis* (Berkeley) saccardo: structure of cordycepic acid. J Am Pharm Assoc 46:114–118
70. Ouyang YY, Zhang Z, Cao YR, Zhang YQ, Tao YY et al (2013) Effects of cordyceps acid and cordycepin on the inflammatory and fibrogenic response of hepatic stellate cells. Chin J Hepatol 21:275–278
71. Wang Y, Yang ZS, Bao DP, Li B, Yin X et al (2021) Improving hypoxia adaption causes distinct effects on growth and bioactive compounds synthesis in an entomopathogenic fungus *cordyceps militaris*. Front Microbiol 12:698436
72. Rao AV, Rao LG (2007) Carotenoids and human health. Hawaii Med J 55:207–216
73. Britton G (1995) Structure and properties of carotenoids in relation to function. FASEB J 9:1551–1558
74. Yang Y, Bu N, Wang S, Zhang J, Wang Y et al (2020) Carotenoid production by caterpillar medicinal mushrooms, *Cordyceps militaris* (Ascomycetes), under different culture conditions. Int J Med Mushrooms 22:1191–1201
75. Dong JZ, Wang SH, Ai XR, Yao L, Sun ZW, Can L (2013) Composition and characterization of cordyxanthins from *cordyceps militaris* fruit bodies. J Funct Food 5:1450–1455
76. Yan XT, Bao HY, Bau T (2010) Isolation and identification of one natural pigment from cultured *Cordyceps militaris*. Mygosystema 29:777–781
77. Yang T, Sun JD, Lian TT, Wang WZ, Dong CH (2014) Process optimization for extraction of carotenoids from medicinal caterpillar fungus, *Cordyceps militaris* (Ascomycetes). Int J Med Mushrooms 16:125–135

78. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G (2013) The hallmarks of aging. Cell 153:1194–1217
79. Tan BL, Norhaizan ME, Liew WPP, Rahman HS (2018) Antioxidant and oxidative stress: a mutual interplay in age-related diseases. Front Pharmacol 9:1162
80. Blount JD, Metcalfe NB, Birkhead TR, Surai PF (2003) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. Science 300:125–127
81. Nagao A (2009) Absorption and function of dietary carotenoids. Forum Nutr 61:55–63
82. Yan XH, Qi QY, Wang SH, Wang CS (2016) Isolation and identification of bioactive compounds from *Cordyceps militaris*. Mycosystema 2016(35):605–610
83. Wang Z, He Z, Li S, Yuan Q (2005) Purification and partial characterization of cu, Zn containing superoxide dismutase from entomogenous fungal species *cordyceps militaris*. Enzym Microb Technol 36:862–869
84. Rukachaisirikul V, Pramjit S, Pakawatchai C, Isaka M, Supothina S (2004) 10-membered macrolides from the insect pathogenic fungus *Cordyceps militaris* BCC 2816. J Nat Prod 67: 1953–1955
85. Jung EC, Kim KD, Bae CH, Kim JC, Kim DK et al (2007) A mushroom lectin from ascomycete *Cordyceps militaris*. Biochim Biophys Acta Gen Subj 1770:833–838
86. Chiu CP, Liu SC, Tang CH, Chan Y, Mohamed ES et al (2016) Anti-inflammatory cerebrosides from cultivated *Cordyceps militaris*. J Agric Food Chem 64:1540–1548
87. Sun JB, Xu J, Wang S, Hou ZD, Lu XC et al (2019a) A new cerebroside from *Cordyceps militaris* with anti-PTP1B activity. Fitoterapia 138:104342
88. Wang J, Chen HL, Li WR, Shan LL (2020) Cordyceps acid alleviates lung cancer in nude mice. J Biochem Mol Toxicol 35:e22670
89. Elkhateeb WA, Daba GM, Thomas P, Wen TC (2019) Medicinal mushrooms as a new source of natural therapeutic bioactive compounds. Egypt Pharm J 18:88–101
90. Huenga DY, Hsieh CH, Cheng YC, Tsai WC, Chen Y (2017) Cordycepin inhibits migration of human glioblastoma cells by affecting lysosomal degradation and protein phosphatase activation. J Nutr Biochem 41:109–116
91. Bawadekji A, Ali KA, Ali MA (2016) A review of the bioactive compound and medicinal value of *Cordyceps militaris*. North J Appl For 1:69–76
92. Liu Y, Wang JH, Wang W, Zhang HY, Zhang XL et al (2015) The chemical constituents and pharmacological actions of *Cordyceps sinensis*. Evid Based Complement Altern Med 2015:1–12
93. Kwon HW, Shin JH, Lim DH, Ok WJ, Nam GS et al (2016) Antiplatelet and antithrombotic effects of cordycepin-enriched WIB-801CE from *Cordyceps militaris* ex vivo, in vivo, and in vitro. BMC Complement Altern Med 16:508
94. Cui JD (2015) Biotechnological production and applications of *Cordyceps militaris*, a valued traditional Chinese medicine. Crit Rev Biotechnol 35:475–484
95. Liao YH, Ling JY, Zhang GY, Liu FJ, Tao S et al (2015) Cordycepin induces cell cycle arrest and apoptosis by inducing DNA damage and up-regulation of p53 in Leukemia cells. Cell Cycle 14:761–771
96. Wu WD, Hu ZM, Shang MJ, Zhao DJ, Huang DS (2014) Cordycepin down-regulates multiple drug resistant (MDR)/HIF-1α through regulating AMPK/mTORC1 signaling in GBC-SD gallbladder cancer cells. Int J Mol Sci 15:12778–12790
97. Lee SK, Lee JH, Kim HR, Chun Y, Lee JH et al (2019) Improved cordycepin production by *Cordyceps militaris* KYL05 using casein hydrolysate in submerged conditions. Biomol Ther 9:461
98. Lee JS, Kwon DS, Lee KR, Park JM, Ha SJ et al (2015) Mechanism of macrophage activation induced by polysaccharide from *cordyceps militaris* culture broth. Carbohydr Polym 120:29–37
99. Kim HS, Kim JY, Kang JS, Kim HM, Kim YO et al (2010) Cordlan polysaccharide isolated from mushroom *Cordyceps militaris* induces dendritic cell maturation through toll-like receptor 4 signalings. Food Chem Toxicol 48:1926–1933

100. Kwon JS, Lee JS, Shin WC, Lee KE, Hong EK (2009) Optimization of culture conditions and medium components for the production of mycelial biomass and exo-polysaccharides with *Cordyceps militaris* in liquid culture. Biotechnol Bioprocess Eng 14:756–762
101. Cheung JH, Li J, Cheung AWH, Yue Z, Zheng KYZ et al (2009) Cordysinocan, a polysaccharide isolated from cultured Cordyceps, activates immune responses in cultured T-lymphocytes and macrophages: Signaling cascade and induction of cytokines. J Ethnopharmacol 124:61–68
102. Else M, Dearden CE, Matutes E, Forconi F, Lauria F et al (2011) Rituximab with pentostatin or cladribine: an effective combination treatment for hairy cell leukemia after disease recurrence. Leuk Lymphoma 52:75–78
103. Li XG, Wu QP, Xie YZ, Ding YR, Du WW et al (2015) Ergosterol purified from medicinal mushroom Amauroderma rude inhibits cancer growth in vitro and in vivo by up-regulating multiple tumor suppressors. Oncotarget 6:17832–17846
104. Yasukawa K, Aoki T, Takido M, Ikekawa T, Saito H et al (1994) Inhibitory effects of ergosterol isolated from the edible mushroom hypsizigus marmoreus on tpa-induced inflammatory ear edema and tumor promotion I mice. Phytother Res 8:10–13
105. Yoo HS, Shin JW, Cho JH, Son CG, Lee YW et al (2004) Effects of *Cordyceps militaris* extract on angiogenesis and tumor growth. Acta Pharmacol Sin 25:657–665
106. Park BT, Na KH, Jung EC, Park JW, Kim HH (2009a) Antifungal and anticancer activities of a protein from the mushroom *Cordyceps militaris*. Korean J Physiol Pharmacol 13:49–54
107. Park SE, Kim J, Lee YW, Yoo HS, Cho CK (2009b) Antitumor activity of water extracts from *Cordyceps militaris* in NCI-H460 cell xenografted nude mice. J Acupunct Meridian Stud 2: 294–300
108. Mollah ML, Dong KP, Park HJ (2012) *Cordyceps militaris* grown on germinated soybean induces G2/M cell cycle arrest through downregulation of cyclin B1 and Cdc25c in human colon cancer HT-29 cells. Evid Based Complement Altern Med 2012:249217
109. Shang XL, Pan LC, Tang Y, Luo Y, Zhu ZY et al (2020) H-1 NMR-based metabonomics of the hypoglycemic effect of polysaccharides from *Cordyceps militaris* on streptozotocin-induced diabetes in mice. Nat Prod Res 34:1366–1372
110. Jo WS, Choi YJ, Kim HJ, Lee JY, Nam BH, Lee JD (2010) The anti-inflammatory effects of water extract from *cordyceps militaris* in murine macrophage. Mycobiology 38:46–51
111. Jin C, Kim G, Choi Y (2008) Induction of apoptosis by aqueous extract of cordyceps militaris through activation of caspases and inactivation of Akt in human breast cancer MDA-MB-231 cells. J Microbiol Biotechnol 18:1997–2003
112. He BL, Zheng QW, Guo LQ, Huang JY, Yun F et al (2020) Structural characterization and immune-enhancing activity of a novel high-molecular-weight polysaccharide from *Cordyceps militaris*. Int J Biol Macromol 145:11–20
113. Rao YK, Fang SH, Wu WS, Tzeng YM (2010) Constituents isolated from *cordyceps militaris* suppress enhanced inflammatory mediator's production and human cancer cell proliferation. J Ethnopharmacol 131:363–367
114. Chu HL, Chien JC, Duh PD (2011) Protective effect of *Cordyceps militaris* against high glucose-induced oxidative stress in human umbilical vein endothelial cells. Food Chem 129: 871–876
115. Nan JX, Park EJ, Yang BK, Song CH, Ko G et al (2001) Antifibrotic effect of extracellular biopolymer from submerged mycelial cultures of *Cordyceps militaris* on liver fibrosis induced by bile duct ligation and scission in rats. Arch Pharm Res 24:327
116. Sun T, Dong W, Jiang G, Yang J, Liu J et al (2019b) *Cordyceps militaris* improves chronic kidney disease by affecting TLR4/NF-κB redox signaling pathway. Oxidative Med Cell Longev 2019:7850863
117. Rupa EJ, Li JF, Arif MH, Yaxi H, Puja AM et al (2020) *Cordyceps militaris* fungus extracts-mediated nanoemulsion for improvement antioxidant, antimicrobial, and anti-inflammatory activities. Molecules 25:5733

118. Tran NKS, Kim GT, Park SH, Lee D, Shim SM et al (2019) Fermented *Cordyceps militaris* extract prevents hepatosteatosis and adipocyte hypertrophy in high fat diet-fed mice. Nutrients 13:50
119. Lin RK, Choong CY, Hsu WH, Tai CJ, Tai CJ (2019) Polysaccharides obtained from mycelia of *Cordyceps militaris* attenuated doxorubicin-induced cytotoxic effects in chemotherapy. Afr Health Sci 19:2156–2163
120. Kim J, Lee H, Kang KS, Chun KH, Hwang GS (2015) *Cordyceps militaris* mushroom and Cordycepin inhibit rankl-induced osteoclast differentiation. J Med Food 18:446–452
121. Hu SM, Wang J, Li FH, Hou PB, Yin JY, Yang ZX (2019) Structural characterisation and cholesterol efflux improving capacity of the novel polysaccharides from *Cordyceps militaris*. Int J Biol Macromol 131:264–272
122. Song QY, Zhu ZY (2020) Using *Cordyceps militaris* extracellular polysaccharides to prevent Pb^{2+}-induced liver and kidney toxicity by activating Nrf2 signals and modulating gut microbiota. Food Funct 11:9226–9239
123. Wu TF, Shi WY, Chiu YC, Chan YY (2021) Investigation of the molecular mechanism underlying the inhibitory activities of ethanol extract of Bombyx mori pupa-incubated *Cordyceps militaris* fruiting bodies toward allergic rhinitis. Biomed Pharmacother 135:111248
124. Yang XQ, Lin P, Wang J, Liu N, Yin F et al (2021) Purification, characterization and anti-atherosclerotic effects of the polysaccharides from the fruiting body of *Cordyceps militaris*. Int J Biol Macromol 181:890–904
125. Lin YE, Chen YC, Lu KH, Huang YJ, Panyod S et al (2021) Antidepressant-like effects of water extract of *Cordyceps militaris* (Linn.) link by modulation of ROCK2/PTEN/Akt signaling in an unpredictable chronic mild stress-induced animal model. J Ethnopharmacol 276: 114194
126. Gallo A, Ferrara M, Perrone G (2013) Phylogenetic study of polyketide synthases and nonribosomal peptide synthetases involved in the biosynthesis of mycotoxins. Toxins 5: 717–742
127. Zheng P, Xia YL, Xiao GH, Xiong CH, Hu X et al (2011) Genome sequence of the insect pathogenic fungus *Cordyceps militaris*, a valued traditional Chinese medicine. Genome Biol 35:475–484
128. Jhou BY, Fang WC, Chen YL, Chen CC (2018) A 90-day subchronic toxicity study of submerged mycelial culture of *Cordyceps militaris* in rats. Toxicol Res 7:977–986
129. Heo JY, Baik HW, Kim HJ, Lee JM, Kim HW et al (2015) The efficacy and safety of *Cordyceps militaris* in Korean adults who have mild liver dysfunction. J Clin Nutr 7:81–86
130. Aramwit P, Porasuphatana S, Srichana T, Nakpheng T (2015) Toxicity evaluation of cordycepin and its delivery system for sustained in vitro anti-lung cancer activity. Nanoscale Res Lett 10:152–161
131. Pintathong P, Chomnunti P, Sangthong S, Jirarat A, Chaiwut P (2021) The feasibility of utilizing cultured *Cordyceps militaris* residues in cosmetics: biological activity assessment of their crude extracts. J Fungi 7:973

8 Lactic Acid Production from Fungal Machineries and Mechanism of PLA Synthesis: Application of AI-Based Technology for Improved Productivity

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Abstract

Lactic acid production and its polymerization to poly-lactide (PLA) using renewable resources have recently gained advancement in the field of biomedical science. It is greeted as a promising alternative to tackle the alarmingly environmental, economical and technological issues raised from excessive use of petroleum-based plastics. PLA due to its good processability and biocompatibility always has fascinated researchers in the clinical sector, yet its high degree of hydrophobicity and absence of reactive groups cause steric hindrance and impeded biofunctionalization of PLA surface for cell attachment. PLA production from renewable resources showed a significant reduction in greenhouse gas

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emissions and fossil energy use as compared to conventional petrochemical-based polymers, thus reducing the threat of global warming. Although lactic acid production from lactic acid bacteria (LAB) is an illustrious domain, production of the green chemical using fungal biomachineries is yet a domain to be explored. Artificial intelligence (AI), a high-tech next-generation technology, is being adapted in all research fields starting from big data analysis to personalized medicines. AI-based mycology modelling for lactic acid production unlocks new prospects for the researcher. The present article is an attempt to explain the potentials of lactic acid production using fungal machineries for sustainable development.

Keywords

Lactic acid · Poly-lactide · Fungal biomachinery · Green chemical · AI-ML

8.1 Introduction

Biopolymer is undoubtedly considered as the material standing between the interphase of modern material science and biology. Over the decades, the devastating outcomes of exploiting petroleum-based polymers forced researchers, academicians, scientists and industrialists to search for alternative eco-friendly polymeric biomaterials. The urge of discovering new biomaterials concomitantly implemented innovative skills and modern knowledge to bring a radical shift in the existing technology, without impinging the 'Mother Nature'. The synergistic approach to utilize renewable sources mostly thrown away as organic wastes by 'microbial machineries' for the production of 'safer' and 'green' chemicals has possibly garnered alarming attention in the domain of both biological and material sciences [1]. In lieu of this, the technological convergence of industrial evolution and biotechnology has already proven the sustainable viability of alternative approaches for the manufacture of green chemicals and biomaterials. Therefore, the paradigm of today's research mostly focuses on developing improved technologies with robust microbial machineries for enhancing the yields and outreach to the consumers at cheaper costs, so that our society can make an absolute turn towards eco-friendly sustainable commodities.

The concept of 'biodegradability' mostly explains the process and capacity to be broken down into simpler forms or products by the action of living entities, mostly microorganisms. The search for biodegradable polymers had led to the introduction of 'biopolymers'. Lactic acid (LA) is one such green chemical, which can be obtained by chemical synthesis or by biotechnological processes via microbes [2]. The monomeric polymerization leads to the formation of poly-lactide (PLA). PLA is one of the highly acclaimed biopolymers, which has gained popularity for its biodegradable and bioabsorbable nature. PLA production from renewable sources showed a significant reduction in greenhouse gas emissions and fossil energy use, in comparison to petro-chemical-based polymers, and thus possibly reduces the threat

of global warming. The biocompatible behaviour of PLA makes it more attractive since it supports the concept of 'Green Sustainable Society'. The induction of degraded products (CO_2 and H_2O) does not interfere in the process of wound healing or regeneration and thus complies with the criteria of an excellent biomaterial for tissue engineering. Although for the last few decades, LA productions have been attempted using lactic acid bacteria (LAB), but to further improve the production system and engross global market, strategies using 'Fungal Machineries' have now been employed. With the advent of science, the precision of the biotechnological process has also been pinching the brains of researchers for making process automatization [3]. The present article is an attempt to explore the possibilities of the use of appropriate fungal machineries and metabolic engineering strategies adopting artificial intelligence (AI)-machine learning (ML) technology for improved LA production, biochemical pathways and transport mechanism including downstream processing. Synthesis of PLA has also been attempted for its biotechnological applications.

8.2 Chemistry of Lactic Acid

LA is an organic acid with a chemical formula of $CH_3CH(OH)CO_2H$. The International Union of Pure and Applied Chemistry (IUPAC) nomenclature for LA is 2-hydroxypropanoic acid. LA is usually naturally produced by a variety of bacteria usually known as LAB [4]. The synthetic form of LA produced in industry also has high value owing to its use in the food industry as an acidity regulator. The weak acidity owes to the partial dissociation in water into H^+ ion and H_3C-CH(OH)-COO^- (lactate ion) radical along with its dissociation constant ($K_a = 1.38 \times 10^{-4}$). The LA in its pure form is colourless and hygroscopic. The conjugate base of LA is called lactate. LA being a chiral compound exhibits its optical nature of L and D isomers when subjected to light scattering. A mixture of two mirror forms of LA (L and D) in equal amounts constitutes the racemic LA. Its backbone is composed of a carbon chain with a central carbon or chiral carbon atom and two carbon atoms at the terminal with a hydroxyl group bonded with the chiral carbon. Terminal sole carbon forms the carboxyl functional group, and the other terminal carbon atom constitutes the methyl group [5]. D or L-LA is miscible with water and ethanol above its melting point (16, 17 or 18 °C) [6]. Basic physico-chemical characteristics of LA are summarized in Table 8.1.

8.3 Production of Lactic Acid

8.3.1 Microbes Involved in Fermentation

Microorganisms play a crucial role in LA production. LA production majorly takes place using two sources, i.e. bacteria and fungi each having unique features [10]. Industrial fermentation of LA with bacteria is mainly carried out in SmF

Table 8.1 Physico-chemical characteristics of lactic acid [7–9]

Characteristic parameter	Description
IUPAC name	2-Hydroxypropanoic acid
Molecular mass	90.08 mol g^{-1}
Physical characteristic	A colourless, viscous liquid with a slight yellowish tint
Taste	Milky acid taste
Odour	Odourless mostly, slightly pungent
Boiling point	122 °C
Melting point	17 °C
Specific gravity	1.2
K_a	$1.38 * 10^{-4}$
pK_a	3.86

(Submerged fermentation) mode. Fungal fermentation (with *Rhizopus oryzae*) has increased attention for the optically pure L (+)-LA production from a varied carbon source (starch, glucose and xylose) in SSF (solid-state fermentation) [11]. From the last few decades, LA production from LAB has been practised on a large scale in industries and also has outstanding contributions to the growth and practice of human civilization. LAB is a generalized term for a wide range of heterogeneous bacteria belonging to diverse taxonomy and morphological physiology, which are gram-positive, mostly non-spore-forming and metabolize carbohydrates to produce LA. In recent years, due to advancement in biotechnological methods, other than the use of wild strains of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Streptococcus*, *Carnobacterium*, *Aerococcus*, *Enterococcus*, *Tetragenococcus*, *Oenococcus*, *Weissella*, *Vagococcus* and *Bifidobacterium* bacteria for LA production, genetically modified strains of bacteria such as *Lactobacillus* spp., *Bacillus* spp., *Corynebacterium glutamicum*, *E. coli*, etc. are also used [12]. Fungal strains such as *Aspergillus niger*, *Rhizopus* spp., *Mucor* spp. and *Monilia* spp. and yeast strains such as *Saccharomyces*, *Candida*, *Zygosaccharomyces* and *Pichia* are used for LA production. Fungal production of LA with the help of strains such as *R. oryzae* has shown improved yield of the desired product and thus has attracted great attention for industrial fermentation. This chapter mainly emphasizes on understanding the LA production using fungal strains.

Fungal members of the genera *Monilia*, *Rhizopus* and *Mucor* are the fungal sources intended for the production of LA [13]. *R. oryzae* among the *Rhizopus* fungi have been considered the most suitable LA-producing strain and thus considered as of great interest. The morphological description of *R. oryzae* defines it as a filamentous, heterothallic, fast-growing microfungus. *Rhizopus* strains show numerous advantage as compared to bacterial fermentation such as low nutrient requirement, cheap downstream processing and further utilization of fungal biomass in the biosorption for contaminated effluent purification and fungal chitosan production [12, 14]. It can synthesize sole isomer L(+)LA from starch obtained from different source such as agro-residue, with their amylolytic enzyme activity. Also, the growth of the fungus is better under nitrogen-limited conditions. On the other hand, the main

drawbacks of fungal fermentation are low yield of LA because of the formation of fumaric acid and ethanol as a by-product and also the need for vigorous aeration that is responsible for the increase in process cost [15]. To overcome the issue of low LA yield, metabolically engineered strains have been constructed. Later, we have discussed genetically modified strains showing high LA yield from a wide variety of starchy feed stocks.

8.3.2 Biochemical Pathways

LA is a natural molecule present in plants, animals and microorganisms. The major production of LA takes place either through chemical synthesis from petroleum products or through the fermentation by microorganisms. The former one yields the racemic mixture of LA and is not specific to any of the optically pure forms, which adds burden to the purification process. The latter one can produce optically active LA and has the advantage of the utilization of sustainable resources, thus making the process environment friendly. Challenges faced for the production of LA using fungal machineries have been discussed in detail. The fungal mycelial layer has lower oxygen concentrations than the dissolved oxygen (DO) level in bulk liquid due to lower oxygen diffusion into the mycelial layer. Therefore, a high DO level (70–90%) is required to achieve the optimum productivity of LA. The highly branched complex structure of fungal mycelia causes rheological problems due to viscous broth, and hence agitation and aeration possess difficulty in conventional agitated tank (SmF) fermenters. To overcome this, two methods were employed: the immobilization technique and bioreactor design [14].

As discussed previously, the chemical production of LA using plant biomass-based feedstock containing crystalline cellulose yields 60% of a racemic mixture of LA with lead as a catalyst [16, 17]. Whereas microbial fermentation is advantageous because of the availability of low-cost sustainable substrates and lesser energy utilization, as fermentation takes place at moderate temperature and does not require additional energy [16, 18]. LA fermentation has been broadly divided into homo (glycolysis and pentose phosphate pathway) and hetero (phosphoketolase pathway)-lactic fermentations. Homo LA fermentation yields LA as only secondary metabolite, but in the hetero LA method, along with lactate other products like aldehyde, ketone, alcohol and organic acids are also produced [19]. Martinez et al. [4] stated that homo LA fermentation is a two-step process: Embden-Meyerhof-Parnas pathway (transformation of glucose into pyruvic acid) comprises the first step, and the second step is the reduction of LA with the aid of reducing power, NADH. Other than LA produced under stress conditions, such as limitation of carbon source or availability of different carbon sources, high pH and low temperature, formic acid is also obtained upon mixed acid fermentation by the activity of the pyruvate-formatelyase enzyme [16, 20]. Key enzymes in glycolysis include glucose-6-P isomerase, 6-phosphofructokinase and fructose bisphosphate aldolase. Substrate-level phosphorylation generates pyruvate from GAP through LA dehydrogenase enzyme, and this pyruvate is reduced to LA along with NAD^+ oxidation [21].

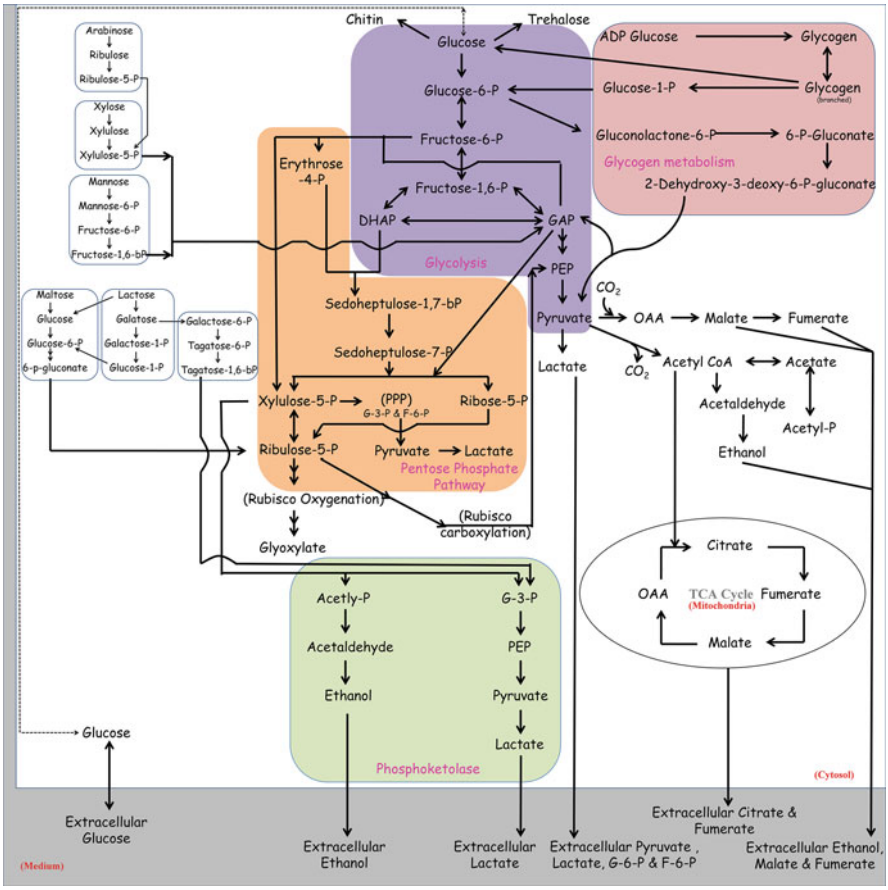

Fig. 8.1 Schematic illustration of the consolidated LA production routes

In pentose phosphate pathway, the 5-C ribulose-5P is produced from a combination of xylulose-5P and ribose-5P (two 5-C compounds) through 6-phosphogluconate dehydrogenase. Transaldolase and transketolase catalyse the formation of sedoheptulose-7P + glyceraldehyde-3P (7 + 3-carbon) and erythrose-4P + fructose-6P (4 + 6 carbon), respectively. Glucose degradation leads to the formation of glyceraldehyde 3-phosphate (to EMP pathway), acetyl-phosphate (ethanol production) and CO_2. In this process, only a fraction of glucose is metabolized via the phosphoketolase (PK) pathway. This process uses the alternate pathway called pentose monophosphate pathway for converting 6-C sugars (hexoses) to 5-C sugars (pentoses) by phosphoketolase [22].

The biochemical production of LA involves the interconnections of various pathways that have been depicted schematically in Fig. 8.1. This schematic includes the biosynthesis of L-LA along with L-malic acid, fumaric acid and ethanol.

Pyruvate acts as a crossroad at the centre, and several key enzymes, including alcohol dehydrogenase (ADH), pyruvate decarboxylase (PDC), lactate dehydrogenase (LDH), pyruvate carboxylase (PC), malate dehydrogenase (MDH) and fumarase, play an important role in the flux determination. These enzymes drive the biochemical reactions, subsequently determining the yield of the desired product and the number of ultimate by-products. Key enzymes in the pentose phosphate pathway include xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulose kinase (XK). The products of PP pathway were further metabolized via the EMP pathway [14].

There are no reports for fungal (wild strain)-derived LA production. *A. niger* co-cultivation with *Lactobacillus* sp. was used for LA production. Saccharification and depolymerization of carbohydrate polymers to fermentable sugars by fungal enzymes are used for the growth of bacteria [23–25]. Finally, to improve the production of LA and to avoid the ethanol or other by-product synthesis, inhibitors of alcohol dehydrogenase (ADH), 2,2,2-trifluoroethanol, are added to fermentation broth being reported as an effective ADH activity inhibitor [26].

8.3.3 Transport Mechanism

To maintain intracellular pH homeostasis (pH 6–8), the microorganism may export the LA either by symport of lactide anion coupled with a proton or by uniport of LA (undissipated) [27]. Two components that maintain electrochemical gradient across the cell are pH gradient and electrochemical potential gradient. The importance of lactate transporter lies in two scenarios: importing lactate anion to utilize it as an energy source and exporting at a high cellular concentration of this molecule to avoid acid stress [28]. Weak acid transport systems are of two types: primary active transport system that uses free biochemical energy (e.g. transport by ATP Binding Cassette protein) and secondary transport system that uses an electrochemical gradient to transfer molecule (e.g. H+/anion antiport) [29, 30]. Most of the microorganisms used in industrial LA production are pH sensitive, and the constant addition of a neutralizing agent is essential. Therefore, understanding the LA transport system will help to develop a superior acid-tolerant strain. The first reported carboxylic acid transporter in yeast was Mae1, a tellurite resistance/dicarboxylate transporter family member associated with malic acid transport [31]. Two lactate-anion transporters that have so far been characterized in fungi are Jen1 and Ady2. The overall transport mechanism of LA in fungi has been described in Fig. 8.2.

8.3.3.1 Jen1

It is one of the best-characterized monocarboxylate transporters which transport lactate, pyruvate, acetate, propionate and micronutrient selenite. It belongs to the major facilitator superfamily (MFS) and shares a common structural topology, i.e. organized into 12 transmembrane segments (TMS) (6 + 6 manner). The role of Jen1 gene for encoding the monocarboxylate lactate-proton symporter in

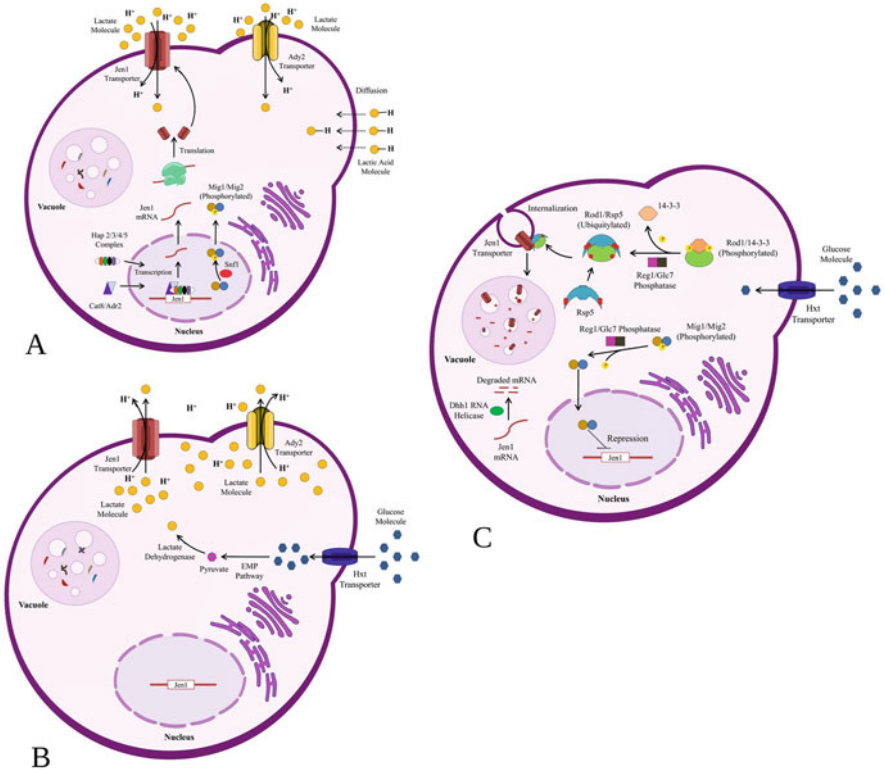

Fig. 8.2 Transport mechanism of LA in fungi. (**a**) Import of lactate in absence of glucose: Snf1 kinase phosphorylates Mig1, and Mig1/Mig2 is transported to cytosol. Hap 2/3/4/5 complex along with Cat8 and Adr2 starts the transcription of JEN1 mRNA followed by the translation. Lactate anion is imported by Jen1 and Ady2 symporter. (**b**) Regulation of lactate transporter in lactate-grown cells upon glucose addition: Rod1 is dephosphorylated by Reg1/Glc7 and released from 14-3-3. Rsp5 directly binds free Rod1 and Rod1/Rsp5 mediates Jen1 internalization. Phosphorylated Mig1/Mig (by Reg1/Glc7) is imported into the nucleus and represses Jen1. Previously synthesized JEN1 mRNA transcript is degraded by Dhh1 helicase. Concurrently, glucose is imported by Hxt transporter. (**c**) Export of lactate: Glucose molecule is converted into lactate via EMP pathway. The concentration of lactate increases inside the cell. Jen1 and Ady2 symporter export lactate to maintain cellular homeostasis

Saccharomyces cerevisiae was first reported in 1999 [32]. Initially, *Jen1* was expected to have an importer function, but several studies suggested that *Jen1* overexpression led to higher LA production [33]. Therefore, *Jen1* should have a bidirectional transport function. Although the crystal structure of *ScJen1* has not yet been solved, but an in silico analysis (based on closed solved the structure of GlpT permease) showed that R188 (2-TMS) plays a critical role in step-wise relocation of the substrate in the protein pore [34]. Rational mutational study of JEN1 identified conserved amino acid sequence ^{379}NXX[S/T]HX[S/T]QDXXXT^{391}, in which replacement even with similar amino acid drastically reduced substrate specificity

and transport capacity. Affinity for all substrates was reduced with Q386N substitution, whereas specific affinity for pyruvate was increased with Q386A substitution [35]. Till now, *CaJen1* and *CaJen2* are only two functionally characterized *ScJen1* orthologues in *Candida albicans*. Phylogenetic analysis of Jen-like sequence in several *Candida* spp. shown to be distributed in two clades: Clade A (containing functionally characterized monocarboxylate transporter *CaJen1*) and Clade B (containing functionally characterized dicarboxylate transporter *CaJen2*). It was assumed that Clade A comprises only monocarboxylate transporter and Clade B comprises only dicarboxylate transporter, but the substrate specificity of both clades was reported to be overlapping. Therefore, it may be possible that the ancestral transporter was able to transport both monocarboxylic and dicarboxylic acid [36]. Expression and regulation of *Jen1* transporter are closely linked with a medium carbon source, i.e. expressed in medium containing LA, acetic acid and pyruvic acid but is repressed in glucose medium [32]. Mig1 and Mig2 are two important DNA-binding repressor proteins that regulate glucose-mediated Jen1 repression [37]. In phosphorylated form, these two proteins are inactive and remain in the cytoplasm. Another protein complex Reg1/Glc7 phosphatase (Reg1, regulatory unit, and Glc7, catalytic unit) dephosphorylates Mig1 and Mig2 transcription factor and is transported to the nucleus where it represses the *Jen1* gene [38]. In presence of lactate, Snf1 kinase phosphorylates Mig1 and inactivates the repressor. Mig1 and Mig2 are translocated back to the cytoplasm, and *Jen1* is derepressed. Transcriptional activators Cat8, Adr1 and Hap2/3/4/5 complex upregulates *Jen1* and other genes associated with non-fermentable carbon sources [28, 39, 40]. At the post-transcriptional level, Jen1 mRNA is also regulated by glucose concentration. 5′-3′ mRNA decay is mediated by Dhh1 helicase and Pat1-Lsm decapping enhancer [41]. The post-translational level of Jen1 regulation is mediated via protein trafficking and endocytosis. The addition of glucose to lactate-grown cells triggers Jen1 endocytosis followed by vacuolar degradation. In presence of LA, Rod1 (an ART family trafficking adaptor) remains phosphorylated and attached to 14-3-3 protein. Upon glucose addition, Glc7/Reg1 phosphatase dephosphorylates Rod1 and detaches from 14-3-3 protein. This dephosphorylated Rod1 now becomes accessible to Rsp5 (an E3 ubiquitin ligase). Rod1/Rsp5 complex now ubiquitylates Jen1 at cell surface followed by internalization [42, 43]. Another alternate mechanism of Jen1 internalization is mediated by α-arrestin Bul1 in response to prolonged growth in lactate [2].

8.3.3.2 Ady2

It is a member of Acetate Uptake Transporter (AceTr) family, originally identified in *S. cerevisiae* as an important gene for sporulation in acetate medium. Like *Jen1*, it also works as a symporter of lactate and plays a vital role in acetate, propionate, formate and pyruvate transport [44, 45]. AceTr members share conserved motifs with NP(A/V/G)P(L/F/V)GL, (Y/F)G(X)FW and NPAPLGL(M/S) amino acid sequence is essential for substrate binding [46, 47]. Although its regulatory mechanism is hardly understood, the role of *Ady2* in LA transport is well documented. Laboratory evolution study on *jen1Δ* mutant in lactate medium showed single-

nucleotide changes in *Ady2* gene. Insertion of this allele induced growth of *jen1Δ ady2Δ* strain indicating the role of *Ady2* gene for lactate transport [44]. Supporting this result, another study showed that constitutive expression of *Ady2* resulted in a high concentration of external LA when glucose was available in the medium [48]. Another interesting study showed that single deletion of *Jen1* or *Ady2* doesn't impact LA production in xylose medium by targeted double-deletion, suggesting a compensating regulation may exist for these two genes [49].

8.3.4 Metabolic Engineering

Metabolic engineering is a robust technology to rewrite cellular metabolism to optimize the production of desired metabolites. With the advancement of '-omics'-based technologies, manipulation of biochemical pathways and integration of new de novo pathways in microbial cell factories have been successfully utilized to produce industrially essential biomolecules [50]. Classical metabolic engineering can be divided into two ways: evolutionary and rational. Evolutionary engineering mimics Darwinism evolution where microorganisms are encouraged to evolve at a much faster rate towards preferable characteristics. In this case, specific understanding of microbial cell factories or metabolic processes is not required. Classical mutagenesis, shotgun mutagenesis, error-prone PCR-based mutagenesis and genome shuffling techniques are commonly used to introduce mutation either in the whole genome or specific gene to generate a mutant population. Then these populations are screened by high-throughput screening methods under a stress condition. On the other hand, rational engineering precisely alters pathways, and microbial cell factories' details are prerequisites. It consists of four steps: learn (analyses all available '-omics' data, computational biology simulation), design (designs a metabolic pathway), build (constructs the system and expression) and test (quantitative/qualitative performance checking and statistical calculations). Rational engineering of LA generally follows two patterns, i.e. pathway redirection and increased gene expression. Pathway redirection aims to tunnel all pyruvate for LA production by deleting competing pathways that use pyruvate to form unwanted metabolites. For example, ethanol is produced from pyruvate by pyruvate decarboxylase (PDC) in yeast, but the pathway redirection strategy mutates PDC to flux all pyruvate for LA. Increased gene expression is an alternative way where the copy number of the desired genes is increased and placed under a suitable promoter. Heterologous gene expression probably is the most common approach for LA metabolic engineering. *Saccharomyces cerevisiae* is not a native producer of LA; hence, from various sources, lactate dehydrogenase has been isolated and expressed in yeast for efficacy testing [51, 52]. Compared to LAB, fungi are better acid-tolerant, are less fastidious and produce optically pure LA. However, ME tools are still required to eliminate several limitations of wild strains like lower LA yield and by-product formation. Here we have discussed three major issues for LA production and its solution. A simplified diagram summarizing evolutionary and rational metabolic engineering approach has been presented in Fig. 8.3.

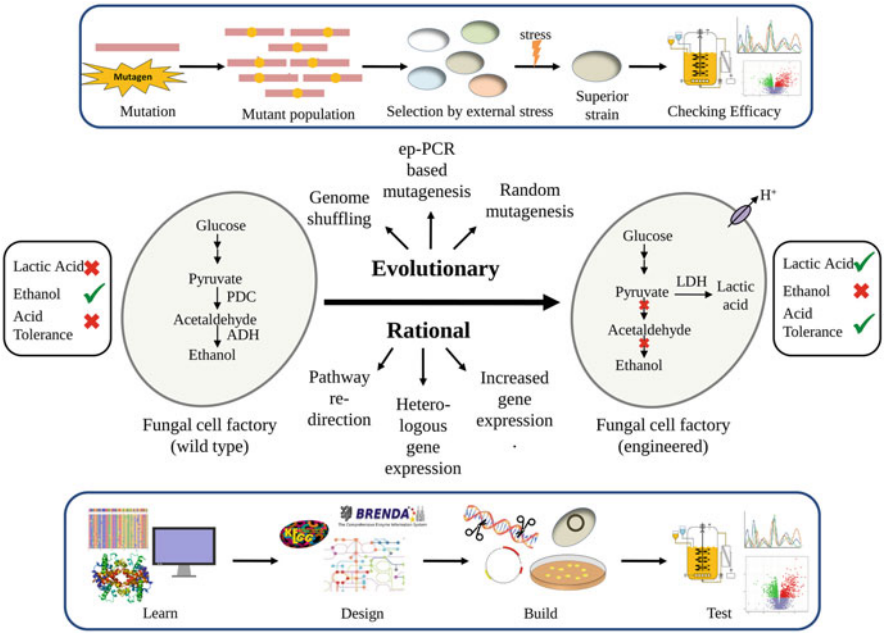

Fig. 8.3 Simplified depiction of metabolic engineering concepts for LA production. Abbreviations used: *Pdc* pyruvate decarboxylase, *Adh* alcohol dehydrogenase, *Ldh* lactate dehydrogenase

8.3.4.1 Acid Tolerance

Earlier studies have demonstrated low pH of the medium stunted the growth of *Saccharomyces cerevisiae* and reduced the yield of LA. According to the external pH, LA (pKa 3.86) will form lactate anion and proton. An influx of these protons may induce inevitable cellular stress like high turgor pressure, protein dysfunction or oxidative stress. To counter this situation, a lump sum amount of energy is depleted to export protons through H + -ATPase and efflux pumps, resulting in growth arrest [53, 54]. In commercial production, lime is continuously added to reduce acidity; however, this practice converts 90% LA into calcium lactate and generates gypsum as a by-product. Hence extra step of acid treatment is required to recover the product, and also gypsum disposal raises an environmental issue. Two monocarboxylate permeases *Jen1* and *Ady2* play a significant role in transporting short-chain monocarboxylic acids (lactate, pyruvate, formate, acetate and propionate) in *S. cerevisiae*. Constitutive expression of *Jen1* or *Ady2* along with lactate dehydrogenase (from *L. casei*) in *S. cerevisiae* W303-1A improved LA production [48]. Similarly, overexpression of *Esbp6* – a mammalian monocarboxylate permease-like protein – showed strong resistance in acidic pH, and LA production increased by 20% [55]. A glycosyl phosphatidyl inositol-anchored membrane protein in *Issatchenkia orientalis* (*IoGas1*) exhibited a vital role in low pH and saline stress adaptation.

However, earlier studies suggested no significant role of Gas1 homolog protein when overexpressed in *S. cerevisiae.* But, recently overexpression of heterologous *IoGas1* gene and disruption of alcohol dehydrogenase (*adh5Δ, adh3Δ, adh4Δ*) and glycerol-3-phosphate dehydrogenase (*gpd1Δ, gpd2Δ*) resulted in D-LA yield up to 0.70 g/g at pH 3.58 [56]. Several transcription factors also have been modulated for better acid tolerance. Haa1 is a well-studied transcription factor that is known to efflux small acids via Tpo2 and Yro2 transporters. Overexpression of *Haa1* in *S. cerevisiae* JHY5330 improved glucose uptake at acidic conditions and increased D-LA yield [57]. *Zygosaccharomyces bailii* is a food spoilage yeast and natural tolerance to low pH has also been engineered to further tune its characteristics. *Ade2* was deleted by PAN-ARS-based CRISPR-Cas9 system, and *R. oryzae* ldhA was introduced in the Pdc1 locus of the host. Engineered strain produced LA 35 g/L with a negligible amount of ethanol [58].

8.3.4.2 Purity

As yeast and other important fungal LA fermenters strongly produce alcohol from pyruvate, this severely affects LA purity. Pyruvate decarboxylase which catalyses the first step of this conversion has been projected as a suitable target. But complete inactivation results in stunted growth and needs to be supplemented by a C2 carbon source. Alcohol dehydrogenase is the second enzyme of ethanol formation that transforms acetaldehyde into ethanol. Deletion of *adh* has also been successful to some extent in reducing ethanol contamination in LA [59]. Poor growth of ethanol pathway mutants is due to redox disbalance. To support the growth of *adh* mutants, Song et al. [60] introduced bacterial acetaldehyde dehydrogenase to complement Ald/Acs pathway in yeast. The engineered strain harbouring heterologous acetyl-CoA pathway produced appreciable LA (142 g/L) with minimal contamination. Zhong et al. [61] systematically modified *D-Ldh* expression step by step to produce highly pure D-LA. They started with *S. cerevisiae* TAMH strain which is a *Pdc*-deficient mutant host and screened different promoters and terminators for *D-Ldh*-containing cassettes. Integration of *D-Ldh* cassette into Ty1 element showed significant improvement. Furthermore, *Jen1, Cyb2, Dld1 and Adh1* were deleted, and the final strain's D-LA production reached 80 g/L.

8.3.4.3 Carbon Source

To reduce the production cost, raw material should be inexpensive and easy to ferment (without pre-treatment) with low contamination rate. Conventional use of corn and refined sugar as starting materials increased the production cost up to 70%. Therefore, various lignocellulosic feedstocks, woody biomass and food waste (from dairy like whey) are being explored to find a suitable substrate for LA fermentation. Yamada et al. [62] used methylotrophic *Pichia pastoris* to produce D-LA from methanol as a whole carbon source. They integrated a bacterial *Ldh* gene into the rDNA spacer region, and resultant strains (GS115/S8/Z3 and GS115/S16/Z3) produced an appreciable amount of D-LA. Alkaline-treated corncob residue containing a large amount of glucose and xylose may be a potential carbon source. *Kluyveromyces marxianus* was engineered in a multistep process: first *Ldh* from a

different source was expressed, then monocarboxylate permease *Jen1* (from *S. cerevisiae*) and 6-phosphofructokinase (*KmPfk*) were overexpressed to increase LA permeation, and finally, *Dld1* was disrupted to minimize LA utilization. Final strain YKX071 produced highly pure D-LA [63]. Woody biomass can be a good choice for microbial fermentation, but it contains lignin (15–35%) as a major component that prevents holocellulose breakdown and negatively affects LA production [64]. Mori et al. [65] engineered white-rot lignin-degrading *Phanerochaete sordida* YK-624. *Ldh* gene from *Bifidobacterium longum* was introduced, and endogenous pdc gene was knocked down by RNA interference. Engineered strain is simultaneously dignified and fermented to produce LA. Few recent reports on application of metabolic engineering have been outlined in Table 8.2.

8.3.5 The Feedstock Used for Lactic Acid Production

LA is the basic ingredient for different product formation in various sectors that includes pharmaceutical, cosmetic, food, agriculture and allied industries, and thus the demand for LA is high at all time. To combat the issue of a high rise in demand, the LA research group has focused on different alternatives such as the use of substrates that are considered as waste, cheap and easily available like dairy waste, agricultural waste, etc., by the use of genetically modified strains, catalyst and different mode of fermentation. In this section, the authors attempted to discuss different substrates being used for the production of LA.

Traditionally, the production of LA is carried out using dairy waste such as whey, skim milk, paneer whey and others, glucose, lactose and starch waste such as potato, wheat, cassava, sorghum and rice from various sources. But with the increase in global demand, the dilemma of food vs fuel was highlighted which leads to an increase in production cost [25]. Therefore, for the synthesis of LA, the focus was shifted to less expensive raw materials. To generate a high quantity of LA at low cost, cheap raw materials, negligible amount of formation of by-products, high productivity and less contamination are mostly preferred, so that the cost for pre-treatment can be reduced. The use of fungal strains is considered as a new approach for the fermentation process other than the traditional bacterial strains. Table 8.3 shows the list of some feedstock that is used for the industrial-scale production of LA by the fungus [12].

8.3.6 Downstream Processing and Recovery

The downstream process for separation and purification is an exceptionally important method for the successful recovery of LA from the effluent. It determines the economic value of the LA to be used for several purposes such as food industries and pharmaceutical areas, etc. An upstream process when fastened with a suitable downstream process provides a more beneficial recovery of LA. Several separation techniques for efficient recovery have been proposed in various literature and

Table 8.2 Few reports on enhanced LA production by metabolic engineering approach

Problem addressed	Microorganism used	Strategy	Outcome	Reference
Acid tolerance	*S. cerevisiae* (host), *L. casei* (*L-Ldh* source)	1. *L. casei L-Ldh* expressed in *S. cerevisiae* W303-1A 2. *Jen1* and *Ady2* constitutively expressed	1. Acid tolerance and yield increased 2. Role of small acid transporter elucidated	[48]
	S. cerevisiae (host), *E. coli* (*Ldh* source), *I. orientalis* (*IoGas1* source)	1. *E. coli Ldh* expressed in *S. cerevisiae* 2. *IoGas1* overexpressed 3. *gpd1Δ, gpd2Δ, adh5Δ, adh3Δ, adh4Δ* mutated	1. Acid tolerance and yield increased 2. D-LA production 92 g/L at pH 3.58	[56]
	S. cerevisiae (host)	*L-Ldh* from bovine and *Esbp6* overexpressed under TDH3 promoter	20% increase in LA production (5.5 g/L) without a non-neutralizing agent	[55]
	S. cerevisiae YPH499/dPdA3-34/DLDH/1-18 recombinant strain (host)	1. Different stress tolerance transcription factors introduced by cocktail δ-integration technique and expression optimized 2. PDR3 transcription factor (related to multi-drug resistance) overexpressed	D-LA production (1.2 times) and yield (1.6 times) increased without a non-neutralizing agent	[66]
Purity	*S. cerevisiae* TAMH (*pdc*-deficient mutant host)	1. 40 *D-Ldh* cassettes screened for best result 2. Suitable promoter and terminator screened. *D-Ldh* cassette integrated into Ty1 TE 4. *jen1*, *cyb2*, *dld1* and *adh1* deleted	1. D-LA production of final strain YIP-J-C-D-AI 80 g/L 2. Optical purity 99.9%	[61]
	S. cerevisiae YPH499 (host), *L. mesenteroides* (*D-Ldh* source)	1. In the first round of global ME, 13 glycolysis genes were expressed and optimized. *pdc1* and *adh1*were deleted 3. In the second round, another 12 glycolysis-related genes were	Double engineered strain YPH499/dPdA3–34/DLDH/1–18 showed an impressive result 1. Optical purity 99.9% 2. Average (of 20 batch fermentation) production ~60 g/L in	[67]

(continued)

Table 8.2 (continued)

Problem addressed	Microorganism used	Strategy	Outcome	Reference
		again expressed and optimized	20 hours 3. Very little ethanol (0.064%) and glycerol (0.026%) produced	
Carbon source	*P. pastoris* (host), *L. mesenteroides* (LDH source)	1. *L. mesenteroides Ldh* gene integrated into rDNA 2. Post-transformation amplification	1. D-LA produced from methanol as carbon source 2. Highest production rate 3.48 g/L in 96 h	[62]
	K. marxianus (host), *B. subtilis* (LDH source), *S. cerevisiae* (ScJEN1 source)	1. Heterologous *Ldh* expressed 2. *ScJen1* and *KmPfk* overexpressed 3. *KmDld1* disrupted	1. D-LA produced from corncob residue 2. Highest productivity 103 g/L at 42 °C with 99.5% purity	[63]
	S. cerevisiae SR8 (host) containing *S. stipitis Xyl1*, *Xyl2* and *Xyl3*	1. Plasmid containing Cas9 introduced in the host 2. Transformant selected 3. PGK1p-LaLDH-CYC1t amplified and introduced in host containing Cas9	1. LA produced from spent coffee grounds 2. Production rate—11.15 g/L after 24 h SSF fermentation in acid-pre-treated whole slurry	[68]

patents. The traditional downstream process produced precipitate in the form of calcium lactate which has to be re-acidified using a strong acid. This generated a large amount of gypsum, making the process unsustainable; also the separation cost was estimated to be 80% of production cost [69, 70]. This indicated an urgent requirement to adopt a downstream process that can produce LA more sustainably and economically feasible [71]. The LA being hydrophilic dimerizes with water, and it becomes unfeasible to obtain pure crystalline LA. This is due to the comparatively large variation in the boiling point of water and LA [72]. However, crystallization in the form of lactate salt after adsorption is feasible.

The classical downstream process involves several processes such as distillation, precipitation, solvent extraction, membrane separation (electrodialysis, reverse osmosis, ultrafiltration process), adsorption, ion exchange, hybrid short path evaporation, etc. [73–75]. Precipitation, acidification, filtration, carbon adsorption, crystallization and evaporation are some of the conventional processes of extraction, while reactive extraction and molecular distillation are the non-conventional processes being used for efficient separation and recovery. The non-conventional approach has gained a huge attraction of researchers over a conventional process to obtain a more efficient, simpler and cheaper method of extraction. These processes

Table 8.3 Recent developments in the usage of feedstock for the production of LA [23]

Organism	Lactic acid concentration (g/L)	Source	Mode	Time
R. arrhizus	46.78	Animal feeds from *Sophora flavescens* residues	Simultaneous saccharification and fermentation (SSF)	48 h
R. microsporus	105–119	Liquefied cassava starch	Fed-batch fermentation	–
R. arrhizus	103.8	Waste potato starch	Bubble column reactor	48 h
Monascus ruber	129–190	Glucose	Fed-batch fermentation	–
R. arrhizus	68.8	Honeycomb matrix Bubble column bioreactor	9 repeated batches	36 days
A. niger	7.7	Glucose	MM supplemented with different glucose concentrations (1–10 percent)	168 h
R. arrhizus UMIP 4.77	10	Wheat straw	Simultaneous saccharification and fermentation (SSF)	48 h
R. oryzae	173.5	Tobacco waste water-extract and glucose	Fed-batch fermentation	120 h
R. oryzae	463.18	Cassava pulp	Solid-state fermentation (SSF)	144 h
R. oryzae	75.28	Cassava pulp hydrolysates	Immobilized cells in the static bed fermentor (batch mode)	96 h
R. oryzae NRRL 395	49.1	Waste paper	Solid-state fermentation	48 h
R. oryzae GY18	80.1	Sucrose	Rotary shaker with agitation rate of 180 rpm	48 h

provide efficient and purified LA yield but possess some disadvantages as well, such as high energy consumption, high-cost input, low solvent recovery, scale-up issues, product purity, etc. Table 8.4 demonstrates the advantages and disadvantages of these separation techniques [73].

The recovery of LA from the fermentation broth is more complex, multistep and difficult than the fermentation process. The number of steps involved in processing also affects the quality and cost factor [76]. The first step in every downstream process is to increase the pH to 10–12 using $Ca(OH)_2$ and temperature to 80–100 °C to disable the microorganism, degrade some of the remaining sugars, coagulate the

Table 8.4 Advantages and disadvantages of separation techniques [73]

Separation techniques	Advantages	Disadvantages
Molecular distillation	– High purification rate – No solvent use – No further purification – Lower the risk of thermal decomposition (due to low evasion temperature and short residence time) – Environment-friendly process	– High vacuum condition required – Difficult to scale up
Precipitation	– Simple operation process – Easy application at industrial scale	– Generation of gypsum that requires landfill disposal – Low product purification rate – High reagent cost – High sulphuric acid consumption – Large amount of wastewater generated
Solvent extraction or liquid-liquid extraction	– Lower the risk of thermal decomposition – No gypsum generation – Flexibility with parameters and physical properties with minimum waste generation	– Low product purity – Unfavourable distribution coefficient obtained by conventional extraction agents – High toxicity of extractants Extraction is regenerated by distillation and stripping (back-extraction)
Membrane separation	High selectivity – High purification rate – Flexibility in large-scale production – Can be merged with conventional fermenters, reducing cost	– High membrane cost – Difficult to scale up – Polarization issues – Membrane fouling issues
Reactive distillation	– Less energy consumption – High purification rate – Reaction and separation can be merged in the same apparatus – Reduced recycling cost – Significant reduction in catalyst amount required for the same degree of conversion	– Complex process – Separation problem by the use of homogeneous catalyst – Corrosion issues – Specifically applied to reversible reaction in the liquid phase

protein and solubilize calcium lactate. Broth acidification is performed to convert lactate into LA, and another cation at pH below 3.86 as above this pH lactate exists in the form of salt. The LA can be separated from other compounds by extraction, ion exchange and precipitation. Martinez et al. [4] reported direct removal of LA without neutralization using membrane separation and adsorption. Furthermore, LA can be concentrated using evaporation, solvent extraction, distillation and crystallization process. The impurities such as sugar residues, acetic/formic acid, etc. can be eliminated by ion exchange and chromatography.

Nanofiltration combined with electrodialysis provides a suitable substitute for the multistep purification process of monomeric LA [77, 78]. Lee et al. [79] proposed the use of two-stage electrodialysis for recovery, and Kim and Moon [80] suggested the use of one-stage electrodialysis with two- or three-compartment water-splitting electrodialysis using a bipolar membrane [69]. However, two-stage electrodialysis was found to be more efficient and sustainable for lactate recovery from fermentation broth. Martins et al. [81] suggested hybrid short path evaporation or molecular distillation procedure for LA recovery and concentration. This process minimizes issues related to thermal decomposition by its short residual time and low evaporation temperature. The use of high operating pressure and refining step reduces the process cost and makes the process technically feasible [82].

Ion exchange chromatography is also extensively used for the separation and successful recovery of pure form of LA. The appropriate selection of anion and cation exchanger makes this process more feasible for separation [4]. Luongo et al. [83] reported IRA-400 (strong) and IR-900 (strong) apt for the recovery of in situ LA. Sorption technology due to its low energy requirement, ease of regeneration and high selectivity is one of the competitive techniques for separation. The adsorption process is influenced by adsorbent properties (matrix, porosity, structure and functional group); adsorbate, i.e. LA; and operating conditions (pH, temperature, contact time, etc.). Lee et al. [79] reported adsorption features of activated carbon and PVP (polyvinyl pyridine) resin for LA purification.

8.3.7 Potential Pathways for Separation and Recovery

Given the several downstream techniques for LA recovery, some potential pathways are being shown in Fig. 8.4. The Fig. 8.4a represent a continuous fermentation process followed by adsorption on ion-exchange resin and desorption using methanol eluent. The major advantage of this process is high purity and recovery yield. The LA solution containing ethanol is further esterified with alcohol. The distillation of ester is followed by hydrolysis, yielding LA and alcohol by-products. Figure 8.4b demonstrates continuous fermentation coupled with reactive distillation, where the extract undergoes further re-extraction followed by esterification and hydrolysis. Reactive extraction and molecular distillation hold promising techniques to increase the concentration of LA [84, 85]. Figure 8.4c represents the classical calcium lactate process. Calcium hydroxide is the cheapest of the alkalis. However, this process is not confined to calcium hydroxides; several other alkalis such as carbonates and hydroxide of other alkali are also being used in several pieces of literature [9]. US Patent 6,384,276 [86] characterized the use of strong acid to liberate the free form of LA. Recovery via precipitation remains unprofitable in terms of environmental and economic aspects as it generates waste residues. Figure 8.4d represents ammonia as neutralizing agent where ammonia reacts with LA-forming ammonium acetate, thereby reducing the acidity of the broth. Ammonia acts as a nutrient for the microorganism in the fermentation process and thereby improves the production rate. The ammonium lactate formed is purified and concentrated using monopolar

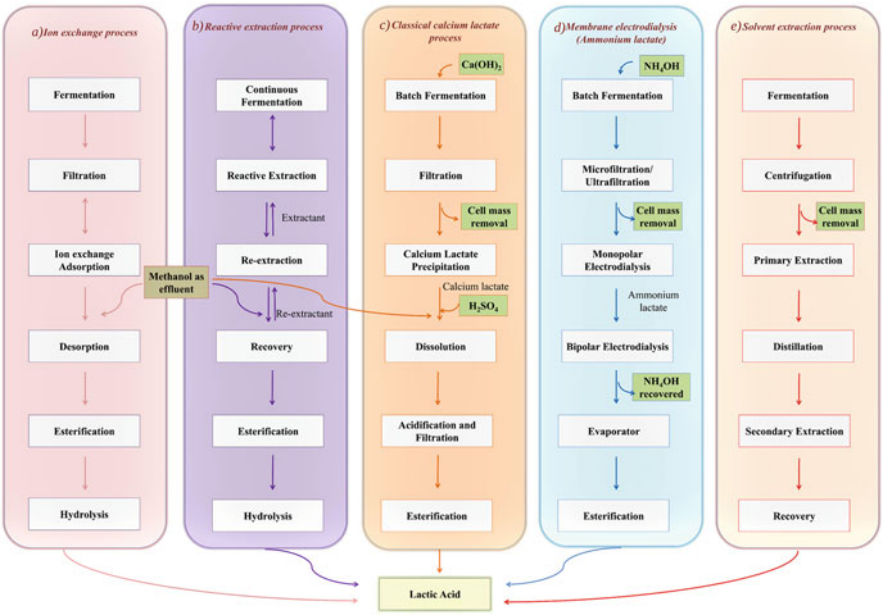

Fig. 8.4 Possible downstream pathway for separation and purification. (**a**) LA recovery using ion exchange process. (**b**) LA recovery using reactive extraction (**c**) Recovery by precipitation- classical calcium lactate process followed by esterification. (**d**) LA recovery by membrane; ammonium lactate process followed by electrodialysis. (**e**) LA recovery using solvent extraction; acidification and esterification

and bipolar electrodialysis producing purified LA. Amide formation at a high temperature can reduce the efficacy of the process. Figure 8.4e represents the continuous acidification followed by esterification with alcohol to form an ester. Filachione et al. [87] suggested esterification for separation and purification where LA in the fermentation broth is esterified with alcohol and catalyst. This alcohol and catalyst can be further separated through distillation followed by hydrolysis to obtain pure LA, but this increases further the cost of the process due to the use of reactor followed by distillation [84]. This process provides optimum purity of the product without any waste generation. But the equipment cost and technical issues in handling fluid with inorganic compounds impart disadvantages to the process.

In recent years, much research has been performed for cost-efficient recovery; still, a much more efficient and economically viable process is required for the finest industrial application as the LA production is still confined by the total production cost that is highly dependent on the downstream processes. Suitable USP may be adopted after the early identification of impurities to minimize the cost of DSP and the overall cost. Accordingly, the theoretical and experimental methods like components, unit operation and optimum conditions determine the development of DSP.

8.4 Bioreactor Design for Lactic Acid Production and Scaling-up

Both the selection of the best bioreactor and the operational settings are important for optimum LA recovery. Over the years, several configurations are used for fungal fermentation: stirred tank reactors, pneumatic reactors, membrane-combined bioreactors, expanded bed and drop column bioreactors, etc. These configurations over time have been modified to improve the productivity, yield and product titre [1, 88]. Stirred tank reactor has been widely used in the commercial fermentation process because of its capacity and industrial reliability [89]. Cultivation of filamentous fungi in typically stirred tank bioreactor maintains homogenous mixing by increasing the agitation speed and also providing high aerobic conditions. The high agitation speed creates high shear stress leading to change in fungi morphology such as biomass formation and mycelial cell growth. The excessive biomass and mycelial growth makes the broth highly viscous; also the mycelia may cling inside the reactor wall, propellers and baffle and may also block the nozzle for aeration, further hindering the process. However, the immobilization of fungal cells came to light by several researchers for troubleshooting the issue [90, 91]. Immobilization was carried out by embedding with natural polymer or by adsorption on polymer support. This immobilization has been investigated with various carrier systems. This improved the stability of the process and also enhanced the enzymatic activity. Thongchul and Yang [92] reported rotating fibrous bed bioreactor (RFBB) to control fungal morphology and improve aeration rate that not only enhanced long-term LA production but also reduced the undesirable ethanol formation.

Pneumatic reactor gained attention in LA production when compared to STR providing the advantage of low energy consumption and uncomplicated process. It includes Bubble Column Reactor (BCR) and Air Lift Reactor (ALR). Du et al. [93] used BCR to circumvent changes in morphology. Zhou et al. [94] utilized BCR for the fermentation process to obtain a high yield with an optimum hydrodynamic condition for biomass transfer. *R. oryzae* pellet can be easily formed by manipulating the initial spore concentration when cultivated in ALRs and BCRs [95, 96].

Silva and Yang [97] proposed a fibrous bed bioreactor (FBB) to integrate with biocatalyst immobilization. FBBs provide a simple and efficient design for both batch and fed-batch fermentation with immobilization support, but the appropriate selection is necessary to remove diffusion limitation between fermentation broth and cells [98, 99]. Chotisubha-Anandha et al. [100] used *R. oryzae* NRRL395 immobilized on a cotton matrix in static FBB and found that aeration and agitation of bioreactor play an important role in LA productivity and *R. oryzae* morphology. The productivity, when compared to the non-immobilized stirred tank reactor, was found to be a maximum of 2.09 g L^{-1} h^{-1}. In a similar study on *R. oryzae* NRRL395 by Pimtong et al. [101], maximum productivity with continuous and batch preparation was 0.72 g L^{-1} h^{-1} and 1.05 g L^{-1} h^{-1}, respectively.

Packed-bed bioreactors (PBRs) are also utilized where biocatalysts are immobilized in the column in pellet or bead form. The fermentation broth is introduced to feed upon biocatalysts along with the support material [102]. The

most recent LA production in PBR was performed in the bioreactor system called 'The Multiferm' multi-fixed-bed bioreactors [103]. The productivity in this bioreactor was 1.5 g L^{-1} h^{-1}. However, only a few studies have been conducted using this reactor. Electrokinetic membrane electrodialysis coupled with fermentation within the bioreactor has also been used in strategies to regulate product inhibition. The electrokinetic bioreactor also has the potential benefit of direct use of hydroxyl ions formed by splitting of water to neutralize pH without any additional acid or base. The potential benefits of the bioreactor include relief of product inhibition and hence an increase in yield, plus separation and concentration of LA and direct use of hydroxyl ions produced by water splitting to neutralize pH without the requirement for additional acid or base. pH control is coupled to the power supply to enable LA to be removed as it is produced.

Another potential bioreactor aimed to improve LA production using membrane bioreactor (MBR). Dorr-Oliver Inc. first reported the crossflow membrane filtration in an activated sludge bioreactor [104, 105]. In this, the membrane is merged with a conventional fermenter allowing simultaneous production and effluent quality in the same unit. MBRs possess several advantages such as various modes of operation (batch, continuous, semi-continuous and immobilized) help to achieve a high level of separation and more purified end product and also enhance anaerobic fermentation of LA by reducing product inhibition. However, the MBR requires stable flux during filtration which can be controlled by inhibiting fouling. The flux stability can be achieved by adjusting the crossflow velocity (CFV) and/or transmembrane pressure (TMP) [104]. The physical and biological characteristics of feed such as morphology, viscosity, temperature and cell density can also be considered for the development of an efficient control strategy [106].

LA productivity can be increased by combining benefits of the multi-stage bioreactor, immobilized matrix with honeycomb structure (asterisk shaped) and membrane bioreactor [17]. The in situ product removal system combined with the reactor configuration not only enables continuous removal of inhibitory metabolites but also prevent product inhibition, minimizing toxicity and improving productivity [107, 108]. A multi-stage reactor can increase productivity and concentration of LA by partly separating the cell growth and production of LA. Kwon et al. [109] utilized a two-stage cell recycle bioreactor arranged in series with the *L. rhamnosus* culture and found successful productivity of 57 and 92 g/L of LA. Kulozik et al. [110] developed a seven-stage cascade cell cycle reactor. The LA productivity of 72 g/L was four times higher than the single-stage reactor. The ceramic (alumina) membrane and polymer (polyacrylonitrile, polysulfone) [111–113] both can be used for the material of membrane bioreactor. The polymer membrane provides flexibility and low-cost production, whereas ceramic membrane provides more chemical and mechanical resistance than polymer membrane [114]. Membrane bioreactor usage eradicates the separate purification process, thereby reducing capital cost and plant footprint. The immobilization matrix can be constructed from stainless steel covered with cotton cloth in an asterisk shape. This honeycomb structure provides a large surface area, void volume and an improved oxygen transfer rate [17]. The bioreactor is equipped with a stainless steel plate containing ports for sampling, pH control, electrodes and an agitation shaft.

Process optimization and scale-up are the important factors to obtain high yield and pure product concentration [115]. It is essential to determine the factors (oxygen transfer rate (OTR), temperature, pH and inoculum) responsible for scaling up the fermentation system. Liu et al. [116] demonstrated that a lower OTR extends the time of fermentation due to limited oxygen supply suggesting the utilization of OTR as a measure for scale-up for L-LA product formation. Miura et al. [95] reported the significant effect of inoculum size on the LA production recommending the regulation of mycelial pellet size by adjusting the initial concentration of spores to 10^6 spores/mL in the pre-culture. They reported a pH of 6.5 to produce the highest production yield. In particular, during the microbial production of enzymes from fungi, pH control has important effects on spore formation, enzyme production and mycelial growth [117, 118]. The optimum temperature has also been proven to have a crucial role in cultural activity. Thus it is important to recognize optimum conditions to scale up the fungi fermentation process for LA production.

Considering these important benefits of bioreactor design and scale-up process, most studies have been focused on the bioreactor design and the scaling-up process to ensure high product yield and meet the latest demand of the industrial application. A consolidated bioreactor design compiling several previously adopted bioreactors [17, 119–121] and theirs parts is presented in Fig. 8.5. To obtain a more efficient

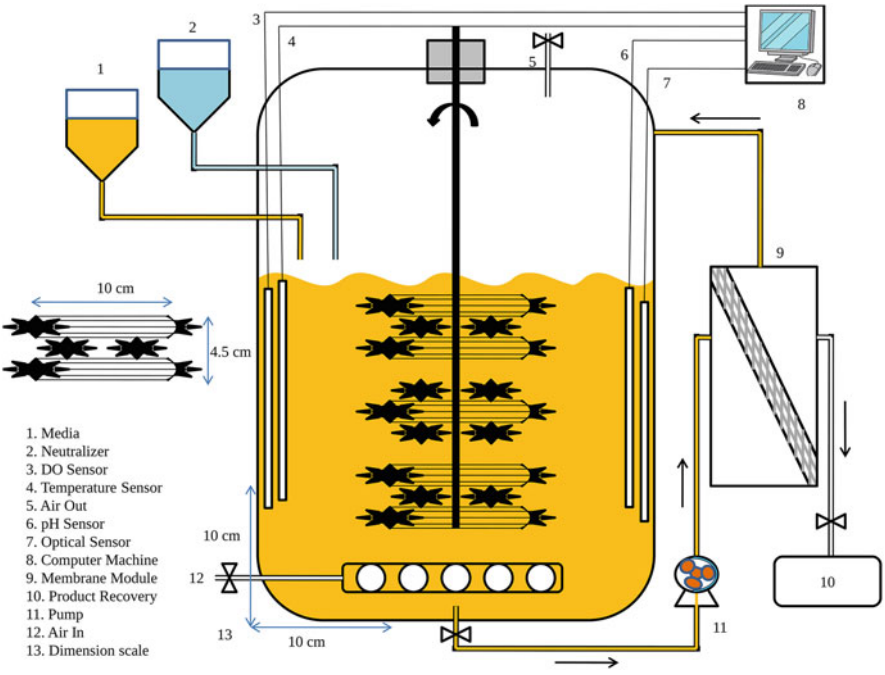

Fig. 8.5 Demonstrate tentative bioreactor design with the increased surface area due to asterisk matrices for the cell immobilization to acquire high LA yield

product yield, an immobilization bioreactor system should be used for large-scale production.

8.5 Overview of Polylactic Acid (PLA) and its Synthesis Methods

8.5.1 PLA: A Green Polymer

In the last several decades, the plastic industry has seen a sharp increase in production and research because of its multidisciplinary application. Simultaneously excessive use of plastics is also the leading cause of white pollution. Several economic and environmental issues arise from petroleum-based polymers, viz. crude oil price volatility, use of non-renewable resource and toxicity to ecosystem. All these concerns led to switch towards bioplastics. PLA is a thermostable, biodegradable aliphatic polyester composed of repeating subunits of 2-hydroxypropanoic acid. Compared to other biopolymers and synthetic polymers, PLA production offers several advantages, e.g.:

I. Cheap and eco-friendly raw materials—its monomer can be obtained from simple microbial fermentation of agricultural biomass [122].
II. Energy-saving conversion of monomer to polymer—PLA can be synthesized with 25–50% less energy expenditure than petroleum-based polymers [123].
III. Biocompatibility—an exciting characteristic of PLA is that it doesn't show any toxic or immunogenic response in local tissue, making it a suitable material for tissue engineering.
IV. Modification—thermo-mechanical characteristics can be tuned as per requirement [124, 125].

With these potentialities to replace petroleum-based polymers, PLA has gained extensive attention in the last two decades, and European Commission also recognized bioplastic as an essential framework for a sustainable and circular economy [126, 127]. Currently, bioplastics contribute less than 1% of total plastic production, but the market revenue of PLA alone is expected to reach 5944.9 million USD by 2027 [128].

Low molecular weight PLA was first reported by Wallace Carothers (Dupont) almost a century ago, who synthesized it by heating LA under vacuum conditions. Later on, they were able to synthesize higher molecular weight PLA, and Vicryl (a PLA-polyglycolic acid blend) was first sold as a commercial product in 1974 [129]. Being a chiral molecule, LA shows two enantiomers, i.e. D-LA and L-LA, and forms three different polymers, i.e. poly(L-LA), poly(D-LA) and poly (D, L-LA). Pure enantiomers show a higher melting temperature and faster crystallization rate than the copolymer of D and L enantiomers. D-lactide confers a more crystalline structure with better thermal and mechanical properties, and 80–90% L-lactide blend is well accepted as good packaging material. The ratio of these two

enantiomers determines crystallinity and mechanical and thermal behaviour of the final product. Glass transition (T_g) is a critical parameter at which drastic change in polymer chain mobility takes place and material transits from glassy to rubbery state. T_g for PLA ranges between 40 and 60 °C, melting temperature (T_m) ranges between 150 and 162 °C, and crystallization temperature (T_C) is 125 °C. Like other non-Newtonian fluids, PLA above its melting temperature acts as a classic flexible chain polymer irrespective of optical compositions [130]. Mechanical and rheological properties also vary depending upon the molecular weight and crystalline nature. Semi-crystalline PLA shows better mechanical characteristics; tensile module, 3 GPa; tensile strength, 50–70 MPa; and flexible strength, 100 MPa. Another desirable property of any biopolymer is easy biodegradability, i.e. 'if there is no biodegradability, there is also no gravity'. PLA shows good biodegradability (takes 2–10 months) than synthetic plastics (takes at least several decades) in natural conditions. Life Cycle Assessment (LCA) indicates the environmental impact of a product starting from raw material to its recycling. Such cradle-to-gate study shows that each ton of PLA emits 1.8 tons less greenhouse gas (GHG) than oil-based plastic, and it is speculated that replacing the global plastic production chain with PLA will reduce 576 million tons of GHG [131, 132].

8.5.2 Synthesis of Polylactic Acid

PLA synthesis is a three-step process—LA production, formation of lactide intermediate and polymerization to get the final product. Although the production of LA monomers from agricultural waste is a viable source, LA can also be produced chemically reacting acetaldehyde and hydrogen cyanide, followed by hydrolysis of lactonitrile, which is the product of the former reaction. Vinyl acetate or glycerol is also used as a precursor molecule to synthesize pure D- or L-LA and racemic mixture [133]. In the overall PLA production process, the synthesis of cyclic lactide is the energy-consuming part. Still, the literature report is limited to patent application and a few industrial protocols only. There are three major schemes for lactide synthesis. In 'two-step synthesis of lactide', LA oligomers are first produced from polycondensation at a vacuum condition and high temperature (130 °C). Then this prepolymer is subjected to depolymerization at 150–240 °C, which results in cyclic lactide. Several catalysts like $Sn(Octate)_2$ and lead oxides are used to remove water smoothly and avoid the production of a racemic mixture [134]. The following option is 'gas-phase synthesis of lactide' where LA vapour is passed through a catalyst bed using inert gas as a carrier. Generally, oxide metals of groups III, IV, V and VIII are used as catalysts. This process offers minimum degradation and racemization, but the volumetric yield is meagre. The third option is the 'one-step liquid-phase process', where water is removed from liquid LA during a ring-closing reaction. Here catalyst like zeolites increases yields >80% and stereospecificity >99% [3, 134]. LA produced either from microbial fermentation or from chemical synthesis is used to synthesize PLA of different molecular weights, but only high MW (>10,000 Da) PLA has industrial importance. Therefore, all approaches of PLA

production aim to maintain stereospecificity and high MW. There are three major routes, i.e. condensation polymerization, azeotropic dehydration condensation and ring-opening polymerization of lactide.

8.5.2.1 Condensation Polymerization

Mitsui Chemicals Co. first successfully synthesized PLA by direct condensation polymerization in 1995 [133]. It is the least expensive path, but it rarely produces solvent-free high MW PLA. There are two different polycondensations—solution polycondensation and melt polycondensation. The overall process can be divided into two steps: first, under high vacuum and temperature, the equimolar concentration of hydroxyl and carboxyl group releases a water molecule and produces low to intermediate MW polymer. In the next step, coupling agents like isocyanates and epoxides extend the chain length. Moon et al. [135] suggested that the initial reaction system is highly polar, but polarity reduces significantly with the progress of the reaction. This polarity change directly affects catalyst activity. A by-product of this route lactide (generated by transesterification reaction) and remaining coupling agents causes severe issues in medical application. Flammable solvent tri-phosgene removes these catalysts and by-products effectively but increases the overall cost and also raises a safety concern [136]. Replacing conventional heating with microwave irradiation, Nagahata et al. produced a high MW PLA (16,000 g/mol) within 30 min [137]. Another report by Cao et al. showed that microwave-assisted heating of LA at 260 °C in the presence of solid super-acids (as a catalyst) generated a low MW PLA (2000 g/mol) within 60 min. Their study also shows that microwave-assisted heating uses 90% less energy than conventional heating [138]. Zahari et al. [139] also explored different power levels of microwaves for PLA polymerization and found that increasing microwave power causes mass loss. Overall microwave-assisted polycondensation is time- and energy-saving but deserves more attention to find an effective way to increase PLA MW. In Fig. 8.6 we have schematically represented exploitation of microbes for LA production and routes of PLA production.

8.5.2.2 Azeotropic Dehydration Condensation

This is a direct method for high MW PLA synthesis without using any adjuvants. Here LA is first distilled in a vacuum condition and high temperature (130 °C) for 2–3 h to remove excess water. Then after adding a catalyst and diphenyl ether, this reaction mixture is passed through a molecular sieve and was continuously recycled for 30–40 h at 130 °C followed by purification. This molecular sieve works as a drying agent to remove water from an organic solvent. Mitsui Chemicals first commercialized this process to generate a long-chain polymer at boiling temperature with aprotic solvent [133, 140]. Kim and Woo [141] azeotropically dehydrated LA and reported a high MW (33,000 g/mol) polymer. They used the different stannous compounds as catalysts and methoxybenzene as the solvent. Recently, Hadiantoro et al. [142] reported a dark grey colour PLA by the same process using xylene solvent and $SnCl_2$ catalyst. Surplus catalyst from the final product is reduced to ppm scale by phosphoric acid treatment.

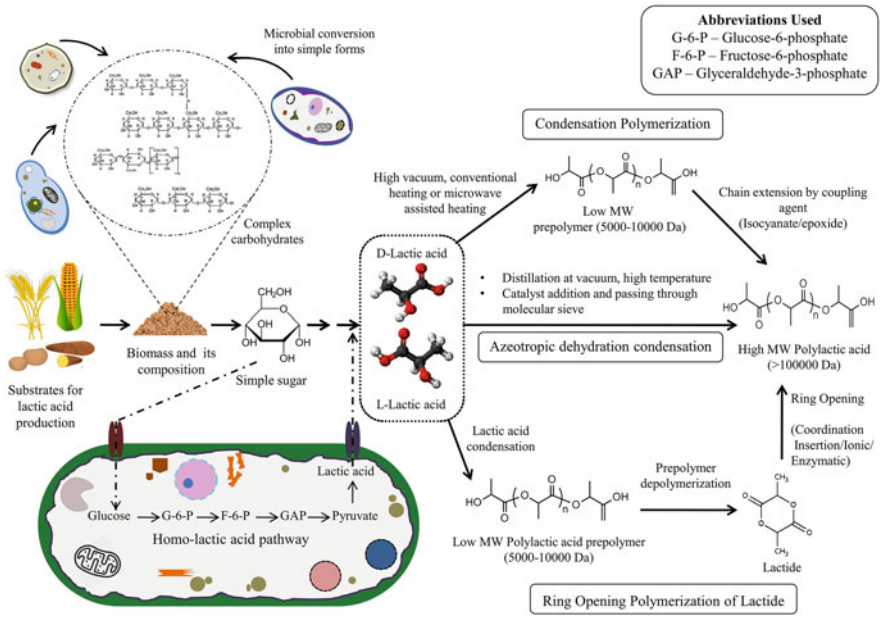

Fig. 8.6 Microbial production of LA and its polymerization into PLA (Modified after Auras et al. [152])

8.5.2.3 Ring-Opening Polymerization

ROP of lactide method is widely used in industrial production because of its reasonable control over optical selectivity and high MW PLA. The overall process is divided into three steps: polycondensation of LA, depolymerization of oligomer (pre-polymer) and ring-opening reaction [143]. This reaction is categorized into coordination insertion and ionic (cationic and anionic) depending on the reaction mechanism. In coordination insertion, the reaction proceeds with metal carboxylate and hydroxyl compounds (like alcohol, polyethene glycol). Nucleophilic alkoxide group on the metal centre first attack oxygen atom of lactide; this forms an intermediate similar to tetrahedral intermediate of carboxylic acid derivative interconversion reaction. Then this intermediate reforms into alkoxide, and a unit of lactide is incorporated in the polymer chain. Propagation continues until cleavage of the metal-alkoxide bond by termination reaction. Stannous octoate and complexes of Al, Mg, Zn, Ti and Ln metals work well to catalyse this reaction [133].

Generally, in cationic ROP, strong acids are used to open the lactide ring. Kricheldorf and Dunsing [144] explored a range of acids and found that triflic acid and methyl triflate can initiate polymerization effectively. Cationic ROP works by the activated monomer (AM) mechanism. Briefly, in this case, the initiator activates the monomer and opens a cyclic ring to form a lactyl alcohol intermediate. Next, this lactyl alcohol attacks a new protonated monomer; thereof, propagation of the chain continues. In terms of safety concerns, this process is superior because acid catalysts

are easily removable and have no issues of toxicity like metal catalysts. A moderate weight PLA was reported by Baśko and Kubisa [145] in a cationic ROP initiated by triflic acid. This reaction was run for 8 h at 120–160 °C using dichloromethane as solvent. An alternative reaction mechanism is the active chain end (ACE), where an oxygen atom of lactide monomer nucleophilically attacks an α-carbon of another monomer attached to an oxonium ion [146]. On the other hand, anionic ROP gained a little attention. A different range of bases has been explored, but only lithium-based complexes like LiCl and LiA_1H_4 have shown acceptable results. Anionic initiator nucleophilically attacks the carbonyl carbon of monomer and opens aryl bond. As a result, lithium alkoxide forms and thereof propagation of the reaction [147]. One drawback of the process is its side reaction and by-product formation.

Enzymatic ROP (e-ROP) is an eco-friendly and viable avenue for PLA synthesis. This approach is emerging in recent days because of its advantages like mild reaction environment, high stereospecificity, inexpensive and no toxicity issue [140]. Lipase has been used as a lactate polymerizing enzyme (LPE). The reaction mechanism is quite simple; lipase interacts with lactate and forms an enzyme-activated-monomer (EM) intermediate. In the subsequent step, a water molecule nucleophilically attacks the aryl carbon of EM intermediate, which initiates the polymerization. In propagation reaction, the terminal –OH group attacks a new EM resulting in the addition of a monomer in each step [148]. Zhao et al. [149] found that the use of low concentration of organic N, N-dimethylacetamide (DMA) or ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF_6]) shows the most promising result. Whulanza et al. [150] performed an e-ROP using *Candida rugosa* lipase (CRL) under different reaction temperatures (70, 90, 110 and 130 °C). The highest MW was recorded at 5428 g/mol with a 93% yield. Bukhari et al. [151] used immobilized *Candida antarctica* lipase B, and microwave-assisted heating was used to maintain a high temperature. However, studies on e-ROP are still at the primary stage. Research needs to be done to find out more efficient natural LPEs or tune existing enzymes for better performance.

8.6 Artificial Intelligence (AI) Strategies for Production Optimization

AI (soft computing) is a machine to view and provide solutions to the problem statement in the same way the field specialist does. AI employs awareness, alertness and apprehension. AI requires a smart piece of code mimicking human action of decision. AI results are consistent and can be documented for future use. It is not an independent working system on its own; it integrates the knowledge and reasoning gained in the past through technological applications, databases and environments. Artificial neural network (ANN), genetic algorithm (GA), particle swarm intelligence (PSI) algorithm, firefly algorithm (FA) and Internet of Things (IoT) are few examples of artificial intelligence that are based on collective behaviour of elements in decentralized and self-organized systems. AI techniques used widely include but are not limited to neural networks, evolutionary computations, fuzzy logic, hybrid

systems, other data mining and machine learning procedures. The tools of AI include chaos theory, fractal analysis, expert (knowledge-based) systems, genetic algorithms and object-oriented programming. AI-based approaches have found various applications and opportunities in biotechnology, agriculture and food science based research activities for multivariate optimization of real complex processes [153]. In biological optimization process, AI-based optimization techniques are gradually getting popularity because of the wide diversity and non-linearity of the system. As biological system is a real complex system, AI-ML-based technologies are popularly been used to address the non-uniform or random iteration among the agents to provide an intelligent behaviour which is unknown to individual agents. This leads to a higher quality of decision-making, minimum amount of uncertainty and a high degree of accuracy in processing a problem using the experience and knowledge that has been accumulated during earlier operations. Because of the above-mentioned reasons, AI is widely adopted in most of the production and manufacturing industries to improve the quality of the product and to minimize error. Successful producers are those updating to the newer technology and employing state-of-the-art technology in their manufacturing [154]. It is a promising multidisciplinary research area and has been widely explored in all sorts of applications.

ANN is having peculiar neuron and neural network and uses mathematical modelling for the interconnections in the network. Training a computer program (ANN) to solve the problem statement involves feeding the experimental data into it as a training set followed by validation and evaluation of the performance of the ANN. Finally, after enough training and validation, the network is capable of calculating the output for the test data, provided the data should have a similar pattern of input used to train the network. The learning rate of the network depends on the coefficient of determination (R^2) for the neural network, and other measures are also used to calculate the performance of the network [155].The learning process is of two types, batch learning and online learning. In batch, a training set is given to learn and then test data is fed to test a neural response. In online learning, the algorithm has to continuously predict while learning [156]. Node organization and establishing the type of correlation between them are called the architecture of network or network topology. Feed-forward, commonly used topology, is (unidirectional from one layer to the next) regarded as an open-loop network since there are no feedback connections [157]. Recurrent architectures, another common network topology, have backward loops, and their output depends upon the previous outputs. Learning algorithms for ANN are of three types called reinforced, supervised and unsupervised algorithms [158]. Multilayer perceptrons (MLPs) are the most used neural networks. Radial-basis-function (RBF), recurrent networks and Kohonen self-organizing neural networks are a few other neural networks [154]. The application of ANN in the optimization of process parameters and bioreactor configurations for LA production using fungi has very limited study. Since it is a new area of ANN application and needs more attention, it would be beneficial to discuss the methodology used in the few ANN-based studies reported for LA production which will provide an insight into the modelling.

To predict the maximum specific growth rate of the ascomycetous fungus *Monascus ruber* in relation to temperature, pH and water activity, a RBF neural network was created and evaluated against a quadratic response surface model. The experimental data was used to test the RBF network and polynomial model. Although given its performance for unseen data, the RBF network delivered very acceptable predictions for the test data set. The most important of the three environmental conditions for fungal growth was found to be temperature, followed by water activity and pH to a lesser amount. Neural network offers a different and more powerful way to represent microbial kinetic features and so might be beneficial in mycology prediction [159].

Ahmad et al. used ANN for culture condition optimization to predict yield of LA and total volatile fatty acid (VFA) production [160]. Here they grow indigenous microbiota on the pre-treated date pulp waste using batch and cyclic mode of operation for LA production. Process parameter optimization to improve the yield of LA and total VFAs production in both cyclic and batch fermentation is complex, hence attempting to achieve by using ANN for forecasting the optimum parameter. Enzymatic or non-enzymatic pre-treated samples along with batch or cyclic mode of fermentation, pH and hydraulic retention time (HRT) were the three input variables. Output variables were LA and total VFAs. Input layer contains pH (X1 = 4, 5, 6, 8), HRT (X2 = 2, 4, 6, 8, 10 days), pre-treated samples (X3 = enzymatic pre-treatment and non-enzymatic pre-treatment), including fermentation processes (batch mode and cyclic mode) as input factors were considered for optimization. Network output comprises LA concentration and total VFAs. The hidden layer comprises ten neurons with sigmoid activation function in a Levenberg-Marquardt backpropagation algorithm. Optimization resulted in LA and VFA concentration of 21.66 and 30.47 (g/L), respectively, in cyclic process with pH 6, using an enzymatically pre-treated sample, and 8 days of HRT at mesophilic condition. Another study by Lunelli et al. [161] in the real-time optimization of LA production from sucrose targeted to optimize the fermentation to avoid the inhibition on the growth of *Lactobacillus plantarum* by the end product (LA) and substrate (sucrose). In this study, a bioreactor model coupled with FORTRAN genetic algorithm (version 1.7a) was developed by Carroll [162]. Tournament strategy is used for selection which allows selection of random pairs for mating with shuffling. Process kinetic parameters were determined using a mathematical model called Luedeking-Piret model for both growth- and non-growth-associated LA production. It is a real-time model for the continuous process. Hence, kinetic model parameters were calculated using GA from the experimental data. The estimated data were used to maximize LA production by achieving an optimal dilution rate.

Neural networks for optically active-LA (L+) production by *R. oryzae* NRRL 395 pellet form have been developed by Dulf et al. Their research aims to make the modelling of LA fermentation by *R. oryzae* NRRL 395 easy even for people who have no experience in any software and neural networks. This was made possible by the development of a user-friendly application (model name, Applicenta). Algorithm coded includes Quasi-Newton, Levenberg-Marquardt, Polak-Ribiere, Scaled Conjugate Gradient and Fletcher-Powell. This application helps to configure the

percentage of data to be split among training, validation and testing along with placeholder to capture several neurons and number of layers. The application development is based on MATLAB® version R2016a. This work has inspired the researcher to create an application for their field of work to simplify the optimization process [163]. These are few examples of ANN-based optimization of LA production, and there are many avenues open in this research area using the above-discussed other methods of AI.

For a complex problem, a better method of AI is needed. Utilizing two or more intelligent methods by combining them is called hybrid systems. A hybrid system of AI methods is more effective in solving problems than an individual method. With the help of an adaptive neuro-fuzzy interference system (ANFIS), the influence of water-silica slurry on polylactic acid (PLA) manufactured by fused deposition modelling (FDM) was investigated under various situations. Controllable factors include building orientation, layer thickness and slurry impact angle. The predictive factors were water weight growth, net weight gain and total weight gain. Different membership functions were used to train ANFIS, which was created in MATLAB software. The ANFIS model was created using three membership functions at each level of controllable factors. The Sugeno fuzzy model was used to create ANFIS fuzzy rules when a generalized bell membership function was adopted. The results show that the ANFIS model was capable of accurately assessing slurry erosion when compared to actual data. The ANFIS and experimental findings were nearly comparable, with an average percentage error of less than 5.45–10.6 [164].

8.6.1 Machine Learning (ML)

Machine learning finds application in management of big data analysis that is generated from different system biology processes (genomics and proteomics), where the application on computational biology and multilayer approaches emerges. Deep learning is an extended arm of machine learning process for handling huge data and its proper analysis; both the techniques are unparallel. ML is a sub-speciality of AI, and it is implemented for the development of methods and techniques which helps the computer to learn. Many techniques and methodologies were developed for ML [165]. To forecast the transformation of a particular product, ML techniques are employed to create a model for the connection between each product and its related reaction rules. Chemical reaction databases such as the Reaxys database, SPRESI database and SOS database can be used to collect training data. Because the training data comes from a chemical reaction database, it may be difficult to locate an appropriate enzyme for each reaction step in the projected route. The 3 N-Monte Carlo Tree Search (MCTS) is a deep neural network based framework created by Segler (an approach which combines three different neural networks and MCTS), which is basically a chemical reaction rules-based retrosynthetic algorithm [166]. Biological system being a real complex system for optimization of multi-variate parameters and their proper interactions for maximization/minimization of product/process development, yield optimization, interaction between each

parameter and their impact on optimization process, AI-ML-based technology is being practised. LA being a microbial product, its further co-polymerization to PLA is being practised. For optimizing the yield of PLA and minimizing the residual unconverted LA, AI-ML-based technologies such as ANN, GA, PSO, etc. are being practised.

8.7 Applications of Polylactic Acid

8.7.1 Medical Sector

8.7.1.1 Tissue Engineering

This is a state-of-the-art technology to restore the structure and integrity of damaged tissue using engineering principles. It starts from a porous scaffold of biomaterials supporting the growth of cells and degradation into nontoxic products after forming well-structured tissue. This scaffold should have surface topology for cell adhesion and migration. It should have the ability to diffuse nutrients and soluble factors and promote the formation of new tissues. PLA having excellent thermal processability, biocompatibility and biodegradability, is an attractive choice for tissue engineering. But few characteristics like brittleness, slow degradability and hydrophobic nature limit their use in the particular scenario only. But copolymerization with other polymers and composite synthesis have been successful in improving these properties [167]. PLA/beta-tricalcium phosphate (TCP) scaffolds were fabricated using rapid volume expansion phase separation techniques. This study showed that the porosity of dense PLA scaffolds was reduced in half, and torsional strength improved after embedding with TCP. Growth factors (VEGF and BMP2)-loaded PLA/β-TCP scaffolds showed 1.8-fold increased neovascularization in quadriceps muscle [168]. Another 3D porous scaffolds were fabricated using natural (collagen, chitosan) and synthetic (PLA mesh) biomaterials to regenerate articular cartilage. Here collagen/chitosan mimicked natural conditions, and PLA provided stiffness. Overall results suggested that collagen-PLA hybrid may be used for fabrication of innovative scaffolds for tissue engineering application [169]. Damage of meniscus tissue is one of the most common problems in orthopaedic science, but healing capacity is limited because of the avascular region. Gupta et al. [170] recently reported PLA and carbohydrate-based hydrogel for meniscal tissue regeneration. In another study efficacy of zeolite-nano-hydroxyapatite-blended poly-epsilon-caprolactone/polylactide nanofibers was checked to revive dental tissues [171]. Overall, PLA-based scaffolds show a promising avenue for tissue engineering.

8.7.1.2 Drug Delivery

Ideal drug carrier aims to introduce the drug in targeted body parts, maintain drug concentration within therapeutic range and avoid side effects. PLA is one of the few FDA-approved polymers with minimum immunogenicity and can be fabricated according to the choice. Other commonly used drug carrier polymers are poly-

epsilon-caprolactone (PCL), poly(glycolic acid) (PGA) and poly(lactic-co-glycolic acid) (PLGA). PLA-based nanoparticles are extensively used because they can maintain a prolonged high concentration release of the drug at the local level. Dong and Feng [172] used a poly (D,L-lactide)/methoxy poly(ethylene glycol)-polylactideblens to deliver paclitaxel, a commonly used antineoplastic drug. Their study showed MPEG/PLA nanoparticles released 99%, whereas pure PLA nanoparticles showed the slowest rate of 37% within 14 days. Electrospun fibres composed of PLA blends are innovative solutions for drug delivery because of their high surface to area ratio and high loading capacity. PolyLA/polybutylene adipate electrospun blend matrices were used to deliver teriflunomide (an anti-rheumatoid drug), and this study showed that higher PBAD concentration results in rapid degradation [173]. Anticancer drugs are also harmful to normal tissues, and the pH of tumour tissues is slightly lower than normal tissues. Here, a pH-sensitive drug release strategy may be a game changer. Yu et al. reported a pH-sensitive polymer-drug conjugate (PDC) of polylactide-graft-doxorubicin (PLA-g-DOX). In vitro result on MCF-7 cell line showed better anticancer efficacy compared to original DOX.HCl [174]. A pH-dependent PLA/DOX/Fe_3O_4 sphere also showed good biocompatibility and promising in vitro results on the HeLa cell line. To synthesize this magnetic PLA sphere, Fe_3O_4 nanoparticles were prepared by co-precipitation with PLA and DOX, followed by coating with oleic acid [175]. Another report suggested that Y-shaped stereo-complexed micelle formed by copolymerization of folic acid-adamantane/β-cyclodextrin-*b*-[poly(D-lactide)]$_2$ (FA-AD/CD-*b*-(PDLA)$_2$) and poly(2-dimethylaminoethyl methacrylate)-*b*-[poly(L-lactide)]$_2$ (PDMAEMA-*b*-(PLLA)$_2$) can act as good nanocarrier [176]. PLA-based nanoparticles are also used as a non-viral vector system for gene delivery but with lower transfection efficacy and a short life span. Chen et al. [177] prepared a methoxypolyethylene glycol-PLA-chitosan (MePEG-PLA-CS) co-polymer nanoparticles which showed better transfection efficacy. In vivo results of MePEG-PLA-CS/DNA conjugate showed that it can withstand GI track of BALb/c mice and promising gene expression. Three-dimensional (3D) printed micro-needles are emerging as an alternate to transdermal drug delivery. Camović et al. [178] fabricated a PLA-based 3D printed microneedle and studied its ability to coat different drug formulations.

8.7.1.3 SARS-CoV2 Management

3D printing technology was already in use, especially in medical science for customizable prototype generation. Vaňková et al. [179] reported a face mask made from PLA using the fused deposition modelling (FDM) technique. Their study clearly mentioned that the PLA face shield structure is sufficient enough to stop SARS-CoV-2 and other harmful microorganisms. Furthermore, these repeated use protective masks can be de-contaminated effectively with conventional surface sterilizing agents, and porosity remains unaffected by mechanical damage. Buluş et al. [180] reported a face mask prepared from PLA-activated charcoal (AC) nanofiber composites using the electrospinning technique. The filtration performance of 10% PLA-8% AC composite was encouraging. Mass testing of the

population also plays a pivotal role in stopping the further spread of highly contagious pathogens like SARS-CoV-2. A PLA-based swab was designed to collect nasopharyngeal samples from suspected patients to meet the increasing demand for testing kits. This cost-effective ($0.05 for each prototype swab) swab had shown sensitivity comparable to commercially available swabs [181].

8.7.2 Non-medical Sector

8.7.2.1 Packaging

The biggest consumer of PLA is the food packaging industry. Danone is one of the first companies to adopt PLA for packaging. They launched a single-use 'Eco Cup' for yoghurt packaging in Germany, and Cargill Dow Polymers LLC provided PLA. Over the last decade, all around the globe, PLA is widely used for packaging short shelf-life foods like vegetables, fruits and meats. The barrier property (i.e. permeation of gas, moisture, and aroma) of a polymer determines its ability to maintain the aroma and freshness of food. Research suggested that a slight change of L:D concentration doesn't affect much; instead, polymer crystallinity is firmly related to barrier properties [123]. The feasibility of PLA for semi-hard cheese has also been explored, and it was found that the transfer of volatile compounds from PLA to cheese was within an acceptable level. But slightly higher lipid oxidation was observed due to porosity [182]. Active food packaging is an innovative idea where packaging polymer releases active agents (antioxidants or antimicrobial compounds) to extend the product's shelf-life. Lukic et al. [183] impregnated thymol and carvacrol on PLA/polycaprolactone films, showing good antioxidant activity against DPPH radicals (lasts for 1 month). Apart from just a packaging polymer, PLA is also projected as an antimicrobial film to control food pathogens in packaged consumables. For example, Jin and Zhang [119] incorporated nisin in PLA film and evaluated its antimicrobial effectiveness. Their data suggested that PLA/nisin film can effectively control *Listeria monocytogenes*, *E. coli* and *Salmonella* sp. Similarly, natural antimicrobial agent rosin was incorporated in PLA/PBAT (polybutylene adipate-co-terephthalate) composites using modified organoclay as compatibilizing agent. These bionanocomposites had shown good tensile strength and exhibited intense antimicrobial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* [184].

8.7.2.2 Agriculture

PLA materials are also replacing traditional agricultural equipment made from synthetic plastics. Exclusion nets act as an alternative way to protect crops from pests and insects. But the surface of commercially available PLA mesh being less hydrophobic in nature allows water ingress and provides a favourable environment for fungal growth. Knoch et al. [185] modified the surface of commercially available PLA and 3D printed PLA mesh by two methods – solvent-free photoinitiated chemical vapour deposition (PICVD) and solvent-induced dip-dip-dry (DDD) method. DDD treatment increased hydrophobicity up to 96–143%, and water ingress

was significantly reduced. They also incorporated limonene in PLA mesh to repel insects and studied preliminary release kinetics. This study concluded that with reduced water ingress and intact mechanical strength, such surface-modified PLA mesh could be a part of Integrated Pest Management. Biodegradable mulches are also becoming part of the intelligent weed control strategy. Herbicide MCPA-PHBV was embedded on PBAT/PLA biofilm and was tested as mulch material to suppress broadleaf weeds with fava bean as a non-target crop. The result indicated that even 1% MCPA concentration was sufficient to stop broadleaf weeds with negligible effect on non-target. But higher herbicide concentration significantly affected non-target. For example, 7.5% and 10% MCPA decreased the quantum yield of photosystem II [186]. But the use of such biodegradable mulches sometimes faces environmental concern issues. Souza et al. [187] investigated the eco-toxicological impact of PBAT film and confirmed no genotoxic potential by comet assay.

8.7.2.3 Textile

PLA fibres share several common characteristics with commercial PET fibres, therefore gaining attention in textile industries also. But poor thermal and poor alkaline hydrolytic resistance is still a problem for PLA fibres. Jabbar et al. [188] prepared PLA/lyocell (PL) blend yarns and knitted fabric followed by a comparison with PET/cotton (PC) blend fabrics. Lyocell proportion raised higher tensile strength and bursting strength in the PL blend. Interestingly, air permeability was increased and water vapour resistance was decreased in the PL blend compared to its counterpart. This characteristic can provide comfort in a hot and humid climate. A similar fabric was prepared from PLA/polyhydroxybutyrate-co-hydroxyvalerate (PLA/PHBV) filament yarns. These PLA/PHBV fabrics exhibited good air permeability, pilling resistance and soft surface. Furthermore, materials showed antimicrobial activity against a few common pathogens, viz. *S. aureus*, *C. albicans* and *Klebsiella pneumoniae* [189].

8.8 Conclusions and Future Perspectives

This chapter outlines the biotechnology and fermentative processes for fungal-derived LA production. Due to widespread application of LA monomer and PLA in medical (tissue engineering, drug delivery and protection masks for SARs CoV-2.) and non-medical (agricultural, packaging and textile) sectors, its demand can't be fulfilled by conventional process. Therefore, exploitation of fungal biomachineries to ferment eco-friendly and cost-effective materials like lignocellulosic biomass is gaining importance. Simultaneously, metabolic engineering is also utilized as an essential tool to enhance acid tolerance, purity and production yield of this biomolecule. The downstream processing of LA from the fermentation broth is another major component of the production cost. Here we have also highlighted the advancements in separation and purification techniques including filtration (micro- and ultrafiltrations), membrane modification for electrodialysis (ED) and bipolar which are gaining industrial importance. The double-ED process is an amalgamation

of desalting and water splitting by bipolar membrane that has gained strong attention for purification and recovery of LA without salt waste generation. Esterification combined with pervaporation of blend of biosolvent has been used as a green solvent to make the process more sustainable and efficient. Hence, from a technological perspective, upgradation and unification of biochemical engineering, bioprocessing, polymer processing, advanced membrane separations and chemical catalysis have brought to light the much efficient technologies for LA production. We have summarized basic biochemistry and transport mechanism of LA, recent advancements in production of LA from non-conventional materials using fungi, downstream processing and finally integration of AI-ML-based techniques for further upregulation of production. However, support from governmental and regulatory policies and consumers are required to overcome the hurdles to bring out sustainable, eco-friendly and low-cost technologies for optimal production of LA.

References

1. Wu H, He AY, Kong XP, Jiang M, Chen XP, Zhu DW, Liu GP, Jin WQ (2015) Acetone–butanol–ethanol production using pH control strategy and immobilized cells in an integrated fermentation–pervaporation process. Process Biochem 50(4):614–622. https://doi.org/10.1016/j.procbio.2014.12.006
2. Talaia G, Gournas C, Saliba E, Barata-Antunes C, Casal M, André B, Diallinas G, Paiva S (2017) The α-arrestin Bul1p mediates lactate transporter endocytosis in response to alkalinization and distinct physiological signals. J Mol Biol 429(23):3678–3695. https://doi.org/10.1016/j.jmb.2017.09.014
3. Balla E, Daniilidis V, Karlioti G, Kalamas T, Stefanidou M, Bikiaris ND, Vlachopoulous A, Koumentakou I, Bikiaris DN (2021) Poly (lactic acid): a versatile biobased polymer for the future with multifunctional properties—from monomer synthesis, polymerization techniques and molecular weight increase to PLA applications. Polymers 13(11):1822. https://doi.org/10.3390/polym13111822
4. Martinez FAC, Balciunas EM, Salgado JM, Gonzalez JMD, Converti A, de Souza Oliveira RP (2013) Lactic acid properties, applications and production: a review. Trends Food Sci Technol 30(1):70–83. https://doi.org/10.1016/j.tifs.2012.11.007
5. Narayanan N, Roychoudhury PK, Srivastava A (2004) L (+) lactic acid fermentation and its product polymerization. Electron J Biotechnol 7(2):167–178. https://doi.org/10.4067/S0717-34582004000200008
6. Chen L, Zeng A, Dong H, Li Q, Niu C (2012) A novel process for recovery and refining of L-lactic acid from fermentation broth. Bioresour Technol 112:280–284. https://doi.org/10.1016/j.biortech.2012.02.100
7. Igoe RS (2011) Dictionary of food ingredients. Springer Science & Business Media
8. Mohanty JN, Das PK, Nanda S, Nayak P, Pradhan P (2015) Comparative analysis of crude and pure lactic acid produced by *lactobacillus fermentum* and its inhibitory effects on spoilage bacteria. The pharma. Innovations 3(11, Part A):38
9. Vaidya AN, Pandey RA, Mudliar S, Kumar MS, Chakrabarti T, Devotta S (2005) Production and recovery of lactic acid for polylactide—an overview. Crit Rev Environ Sci Technol 35(5):429–467. https://doi.org/10.1080/10643380590966181
10. Komesu A, de Oliveira JAR, da Silva Martins LH, Maciel MRW, Maciel Filho R (2017) Lactic acid production to purification: a review. Bioresources 12(2):4364–4383

11. Wang L, & Yang ST (2007) Solid state fermentation and its applications. Bioprocessing for value-added products from renewable resources, 465-489. DOI:https://doi.org/10.1016/B978-044452114-9/50019-0
12. Panesar PS, Kaur S (2015) Bioutilisation of agro-industrial waste for lactic acid production. Int J Food Sci Technol 50(10):2143–2151. https://doi.org/10.1111/ijfs.12886
13. Gleason FH, Price JS (1969) Lactic acid fermentation in lower fungi. Mycologia 61(5): 945–956. https://doi.org/10.1080/00275514.1969.12018817
14. Zhang ZY, Jin B, Kelly JM (2007) Production of lactic acid from renewable materials by *Rhizopus* fungi. Biochem Eng J 35(3):251–263. https://doi.org/10.1016/j.bej.2007.01.028
15. Krishna BS, Nikhilesh GSS, Tarun B, Saibaba N, Gopinadh R (2018) Industrial production of lactic acid and its applications. Int J Biotechnol Res 1:42–54
16. Gao C, Ma C, Xu P (2011) Biotechnological routes based on lactic acid production from biomass. Biotechnol Adv 29(6):930–939. https://doi.org/10.1016/j.biotechadv.2011.07.022
17. Wang Z, Wang Y, Yang ST, Wang R, Ren H (2010) A novel honeycomb matrix for cell immobilization to enhance lactic acid production by *Rhizopus oryzae*. Bioresour Technol 101(14):5557–5564. https://doi.org/10.1016/j.biortech.2010.02.064
18. Okano K, Tanaka T, Ogino C, Fukuda H, Kondo A (2010) Biotechnological production of enantiomeric pure lactic acid from renewable resources: recent achievements, perspectives, and limits. Appl Microbiol Biotechnol 85(3):413–423. https://doi.org/10.1007/s00253-009-2280-5
19. Das M, Kundu D, Rastogi A, Singh J, Banerjee R (2018) Biotechnological exploitation of poly-lactide produced from cost effective lactic acid. Principles and applications of fermentation technology, vol 18. Wiley, Hoboken, NJ, pp 401–416. https://doi.org/10.1002/9781119460381.ch18
20. Mayo B, Aleksandrzak-Piekarczyk T, Fernández M, Kowalczyk M, Álvarez-Martín P, & Bardowski J (2010) Updates in the metabolism of lactic acid bacteria. Biotechnology of lactic acid bacteria: Novel applications, 3-33. https://doi.org/10.1002/9780813820866.ch1
21. Juturu V, Wu JC (2016) Microbial production of lactic acid: the latest development. Crit Rev Biotechnol 36(6):967–977. https://doi.org/10.3109/07388551.2015.1066305
22. Abdel-Rahman MA, Tashiro Y, Sonomoto K (2013) Recent advances in lactic acid production by microbial fermentation processes. Biotechnol Adv 31(6):877–902. https://doi.org/10.1016/j.biotechadv.2013.04.002
23. Abedi E, Hashemi SMB (2020) Lactic acid production–producing microorganisms and substrates sources-state of art. Heliyon 6(10):e04974. https://doi.org/10.1016/j.heliyon.2020.e04974
24. Ge XY, Qian H, Zhang WG (2009) Improvement of l-lactic acid production from Jerusalem artichoke tubers by mixed culture of *Aspergillus niger* and *Lactobacillus* sp. Bioresour Technol 100(5):1872–1874. https://doi.org/10.1016/j.biortech.2008.09.049
25. John RP, Anisha GS, Nampoothiri KM, Pandey A (2009) Direct lactic acid fermentation: focus on simultaneous saccharification and lactic acid production. Biotechnol Adv 27(2): 145–152. https://doi.org/10.1016/j.biotechadv.2008.10.004
26. Thitiprasert S, Sooksai S, Thongchul N (2011) In vivo regulation of alcohol dehydrogenase and lactate dehydrogenase in *Rhizopus oryzae* to improve L-lactic acid fermentation. Appl Biochem Biotechnol 164(8):1305–1322. https://doi.org/10.1007/s12010-011-9214-2
27. van Maris AJ, Konings WN, van Dijken JP, Pronk JT (2004) Microbial export of lactic and 3-hydroxypropanoic acid: implications for industrial fermentation processes. Metab Eng 6(4): 245–255. https://doi.org/10.1016/j.ymben.2004.05.001
28. Casal M, Queirós O, Talaia G, Ribas D, & Paiva S (2016) Carboxylic acids plasma membrane transporters in *Saccharomyces cerevisiae*. Yeast membrane transport, 229-251.DOI: https://doi.org/10.1007/978-3-319-25304-6_9
29. Hettema EH, Van Roermund CW, Distel B, van den Berg M, Vilela C, Rodrigues-Pousada C, Wanders RJ, Tabak HF (1996) The ABC transporter proteins Pat1 and Pat2 are required for

import of long-chain fatty acids into peroxisomes of *Saccharomyces cerevisiae*. EMBO J 15(15):3813–3822. https://doi.org/10.1002/j.1460-2075.1996.tb00755.x
30. Okorokov LA, Kulakovskaya TV, Lichko LP, Polorotova EV (1985) H+/ion antiport as the principal mechanism of transport systems in the vacuolar membrane of the yeast *Saccharomyces carlsbergensis*. FEBS Lett 192(2):303–306. https://doi.org/10.1016/0014-5793(85)80130-7
31. Grobler J, Bauer F, Subden RE, Van Vuuren HJ (1995) The mae1 gene of Schizosaccharomyces pombe encodes a permease for malate and other C4 dicarboxylic acids. Yeast 11(15):1485–1491. https://doi.org/10.1002/yea.320111503
32. Casal M, Paiva S, Andrade RP, Gancedo C, Leão C (1999) The lactate-proton symport of *Saccharomyces cerevisiae* is encoded by JEN1. J Bacteriol 181(8):2620–2623. https://doi.org/10.1128/JB.181.8.2620-2623.1999
33. Wakamatsu M, Tomitaka M, Tani T, Taguchi H, Kida K, Akamatsu T (2013) Improvement of ethanol production from D-lactic acid by constitutive expression of lactate transporter Jen1p in *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 77(5):1114–1116. https://doi.org/10.1271/bbb.120985
34. Soares-Silva I, Sá-Pessoa J, Myrianthopoulos V, Mikros E, Casal M, Diallinas G (2011) A substrate translocation trajectory in a cytoplasm-facing topological model of the monocarboxylate/H+ symporter Jen1p. Mol Microbiol 81(3):805–817. https://doi.org/10.1111/j.1365-2958.2011.07729.x
35. Soares-Silva I, Paiva S, Diallinas G, Casal M (2007) The conserved sequence NXX [S/T] HX [S/T] QDXXXT of the lactate/pyruvate: H+ symporter subfamily defines the function of the substrate translocation pathway. Mol Membr Biol 24(5–6):464–474. https://doi.org/10.1080/09687680701342669
36. Alves R, Sousa-Silva M, Vieira D, Soares P, Chebaro Y, Lorenz MC, Casai M, Soares-Silva, Paiva S (2020) Carboxylic acid transporters in *Candida* pathogenesis. MBio 11(3):e00156–e00120. https://doi.org/10.1128/mBio.00156-20
37. Andrade RP, Casal M (2001) Expression of the lactate permease gene JEN1 from the yeast *Saccharomyces cerevisiae*. Fungal Genet Biol 32(2):105–111. https://doi.org/10.1006/fgbi.2001.1254
38. Sanz P, Alms GR, Haystead TA, Carlson M (2000) Regulatory interactions between the Reg1-Glc7 protein phosphatase and the Snf1 protein kinase. Mol Cell Biol 20(4):1321–1328. https://doi.org/10.1128/MCB.20.4.1321-1328.2000
39. Haurie V, Perrot M, Mini T, Jenö P, Sagliocco F, Boucherie H (2001) The transcriptional activator Cat8p provides a major contribution to the reprogramming of carbon metabolism during the diauxic shift in *Saccharomyces cerevisiae*. J Biol Chem 276(1):76–85. https://doi.org/10.1074/jbc.M008752200
40. Olesen JT, Guarente L (1990) The HAP2 subunit of yeast CCAAT transcriptional activator contains adjacent domains for subunit association and DNA recognition: model for the HAP2/3/4 complex. Genes Dev 4(10):1714–1729. https://doi.org/10.1101/gad.4.10.1714
41. Mota S, Vieira N, Barbosa S, Delaveau T, Torchet C, Le Saux A, Garcia M, Pereira A, Lemoine S, Couipier F, Darzacq X, Benard L, Casal M, Devaux F, Paiva S (2014) Role of the DHH1 gene in the regulation of monocarboxylic acids transporters expression in *Saccharomyces cerevisiae*. PLoS One 9(11):e111589. https://doi.org/10.1371/journal.pone.0111589
42. Becuwe M, Vieira N, Lara D, Gomes-Rezende J, Soares-Cunha C, Casal M, Haguenauer-Tsapis R, Vincent O, Paiva S, Léon S (2012) A molecular switch on an arrestin-like protein relays glucose signalling to transporter endocytosis. J Cell Biol 196(2):247–259. https://doi.org/10.1083/jcb.201109113
43. Paiva S, Vieira N, Nondier I, Haguenauer-Tsapis R, Casal M, Urban-Grimal D (2009) Glucose-induced ubiquitylation and endocytosis of the yeast Jen1 transporter: role of lysine 63-linked ubiquitin chains. J Biol Chem 284(29):19228–19236. https://doi.org/10.1074/jbc.M109.008318

44. de Kok S, Nijkamp JF, Oud B, Roque FC, de Ridder D, Daran JM, Pronk JT, van Maris AJ (2012) Laboratory evolution of new lactate transporter genes in a jen1 Δ mutant of Saccharomyces cerevisiae and their identification as ADY2 alleles by whole-genome resequencing and transcriptome analysis. FEMS Yeast Res 12(3):359–374. https://doi.org/10.1111/j.1567-1364.2011.00787.x
45. Paiva S, Devaux F, Barbosa S, Jacq C, Casal M (2004) Ady2p is essential for the acetate permease activity in the yeast *Saccharomyces cerevisiae*. Yeast 21(3):201–210. https://doi.org/10.1002/yea.1056
46. Augstein A, Barth K, Gentsch M, Kohlwein SD, Barth G (2003) Characterization, localization and functional analysis of Gpr1p, a protein affecting sensitivity to acetic acid in the yeast *Yarrowia lipolytica*. Microbiology 149(3):589–600. https://doi.org/10.1099/mic.0.25917-0
47. Ribas D, Soares-Silva I, Vieira D, Sousa-Silva M, Sa-Pessoa J, Azevedo-Silva J, Viegas SC, Arraiano CM, Diallinas G, Paiva S, Soares P, Casal M (2019) The acetate uptake transporter family motif "NPAPLGL (M/S)" is essential for substrate uptake. Fungal Genet Biol 122:1–10. https://doi.org/10.1016/j.fgb.2018.10.001
48. Pacheco A, Talaia G, Sá-Pessoa J, Bessa D, Gonçalves MJ, Moreira R, Paiva S, Casal M, Queirós O (2012) Lactic acid production in *Saccharomyces cerevisiae* is modulated by expression of the monocarboxylate transporters Jen1 and Ady2. FEMS Yeast Res 12(3):375–381. https://doi.org/10.1111/j.1567-1364.2012.00790.x
49. Turner TL, Lane S, Jayakody LN, Zhang GC, Kim H, Cho W, Jin YS (2019) Deletion of JEN1 and ADY2 reduces lactic acid yield from an engineered Saccharomyces cerevisiae, in xylose medium, expressing a heterologous lactate dehydrogenase. FEMS Yeast Res 19(6):foz050. https://doi.org/10.1093/femsyr/foz050
50. Singh SK, Ahmed SU, Pandey A (2006) Metabolic engineering approaches for lactic acid production. Process Biochem 41(5):991–1000. https://doi.org/10.1016/j.procbio.2005.12.004
51. Upadhyaya BP, DeVeaux LC, Christopher LP (2014) Metabolic engineering as a tool for enhanced lactic acid production. Trends Biotechnol 32(12):637–644. https://doi.org/10.1016/j.tibtech.2014.10.005
52. Wakai S, Arazoe T, Ogino C, Kondo A (2017) Future insights in fungal metabolic engineering. Bioresour Technol 245:1314–1326. https://doi.org/10.1016/j.biortech.2017.04.095
53. Narendranath NV, Thomas KC, Ingledew WM (2001) Effects of acetic acid and lactic acid on the growth of *Saccharomyces cerevisiae* in a minimal medium. J Ind Microbiol Biotechnol 26(3):171–177. https://doi.org/10.1038/sj.jim.7000090
54. Peetermans A, Foulquié-Moreno MR, Thevelein JM (2021) Mechanisms underlying lactic acid tolerance and its influence on lactic acid production in *Saccharomyces cerevisiae*. Microbial. Cell 8(6):111. https://doi.org/10.15698/mic2021.06.751
55. Sugiyama M, Akase SP, Nakanishi R, Kaneko Y, Harashima S (2016) Overexpression of ESBP6 improves lactic acid resistance and production in *Saccharomyces cerevisiae*. J Biosci Bioeng 122(4):415–420. https://doi.org/10.1016/j.jbiosc.2016.03.010
56. Zhong W, Yang M, Hao X, Sharshar MM, Wang Q, Xing J (2020) Improvement of D-lactic acid production at low pH through expressing acid-resistant gene IoGAS1 in engineered *Saccharomyces cerevisiae*. J Chem Technol Biotechnol 96(3):732–742. https://doi.org/10.1002/jctb.6587
57. Baek SH, Kwon EY, Kim YH, Hahn JS (2015) Metabolic engineering and adaptive evolution for efficient production of D-lactic acid in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 100(6):2737–2748. https://doi.org/10.1007/s00253-015-7174-0
58. Kuanyshev N, Rao CV, Dien B, Jin YS (2020) Domesticating a food spoilage yeast into an organic acid-tolerant metabolic engineering host: lactic acid production by engineered *Zygosaccharomyces bailii*. Biotechnol Bioeng 118(1):372–382. https://doi.org/10.1002/bit.27576
59. Flikweert MT, de Swaaf M, van Dijken JP, Pronk JT (1999) Growth requirements of pyruvate-decarboxylase-negative Saccharomyces cerevisiae. FEMS Microbiol Lett 174(1):73–79. https://doi.org/10.1111/j.1574-6968.1999.tb13551.x

60. Song JY, Park JS, Kang CD, Cho HY, Yang D, Lee S, Cho KM (2016) Introduction of a bacterial acetyl-CoA synthesis pathway improves lactic acid production in *Saccharomyces cerevisiae*. Metab Eng 35:38–45. https://doi.org/10.1016/j.ymben.2015.09.006
61. Zhong W, Yang M, Mu T, Wu F, Hao X, Chen R, Sharshar MM, Thygesen A, Wang Q, Xing J (2019) Systematically redesigning and optimizing the expression of D-lactate dehydrogenase efficiently produces high-optical-purity D-lactic acid in *Saccharomyces cerevisiae*. Biochem Eng J 144:217–226. https://doi.org/10.1016/j.bej.2018.09.013
62. Yamada R, Ogura K, Kimoto Y, Ogino H (2019) Toward the construction of a technology platform for chemicals production from methanol: D-lactic acid production from methanol by an engineered yeast *Pichia pastoris*. World J Microbiol Biotechnol 35(2):37. https://doi.org/10.1007/s11274-019-2610-4
63. Kong X, Zhang B, Hua Y, Zhu Y, Li W, Wang D, Hong J (2019) Efficient L-lactic acid production from corncob residue using metabolically engineered thermo-tolerant yeast. Bioresour Technol 273:220–230. https://doi.org/10.1016/j.biortech.2018.11.018
64. Gomes D, Rodrigues AC, Domingues L, Gama M (2015) Cellulase recycling in biorefineries is it possible? Appl Microbiol Biotechnol 99(10):4131–4143. https://doi.org/10.1007/s00253-015-6535-z
65. Mori T, Kako H, Sumiya T, Kawagishi H, Hirai H (2016) Direct lactic acid production from beech wood by transgenic white-rot fungus *Phanerochaete sordida* YK-624. J Biotechnol 239:83–89. https://doi.org/10.1016/j.jbiotec.2016.10.014
66. Yamada R, Kumata Y, Mitsui R, Matsumoto T, Ogino H (2021) Improvement of lactic acid tolerance by cocktail δ-integration strategy and identification of the transcription factor PDR3 responsible for lactic acid tolerance in yeast *Saccharomyces cerevisiae*. World J Microbiol Biotechnol 37(2):1–10. https://doi.org/10.1007/s11274-020-02977-1
67. Yamada R, Wakita K, Mitsui R, Ogino H (2017) Enhanced d-lactic acid production by recombinant *Saccharomyces cerevisiae* following optimization of the global metabolic pathway. Biotechnol Bioeng 114(9):2075–2084. https://doi.org/10.1002/bit.26330
68. Kim JW, Jang JH, Yeo HJ, Seol J, Kim SR, Jung YH (2019) Lactic acid production from a whole slurry of acid-pretreated spent coffee grounds by engineered *Saccharomyces cerevisiae*. Appl Biochem Biotechnol 189(1):206–216. https://doi.org/10.1007/s12010-019-03000-6
69. Hábová V, Melzoch K, Rychtera M, Sekavová B (2004) Electrodialysis as a useful technique for lactic acid separation from a model solution and a fermentation broth. Desalination 162:361–372. https://doi.org/10.1016/S0011-9164(04)00070-0
70. Joglekar HG, Rahman I, Babu S, Kulkarni BD, Joshi A (2006) Comparative assessment of downstream processing options for lactic acid. Sep Purif Technol 52(1):1–17. https://doi.org/10.1016/j.seppur.2006.03.015
71. Hulse GK, Stalenberg V, McCallum D, Smit W, O'neil G, Morris N, Tait RJ (2005) Histological changes over time around the site of sustained release naltrexone-poly (DL-lactide) implants in humans. J Control Release 108(1):43–55. https://doi.org/10.1016/j.jconrel.2005.08.001
72. Lunelli BH, Andrade RR, Atala DI, Maciel MRW, Maugeri Filho F, Maciel Filho R (2010) Production of lactic acid from sucrose: strain selection, fermentation, and kinetic modeling. Appl Biochem Biotechnol 161(1):227–237. https://doi.org/10.1007/s12010-009-8828-0
73. Komesu A, Wolf Maciel MR, Rocha de Oliveira JA, da Silva Martins LH, Maciel Filho R (2017) Purification of lactic acid produced by fermentation: focus on non-traditional distillation processes. Sep Purif Rev 46(3):241–254. https://doi.org/10.1080/15422119.2016.1260034
74. Krzyżaniak A, Leeman M, Vossebeld F, Visser TJ, Schuur B, de Haan AB (2013) Novel extractants for the recovery of fermentation derived lactic acid. Sep Purif Technol 111:82–89. https://doi.org/10.1016/j.seppur.2013.03.031
75. Nakano S, Ugwu CU, Tokiwa Y (2012) Efficient production of d-(−)-lactic acid from broken rice by *lactobacillus delbrueckii* using ca(OH)2 as a neutralizing agent. Bioresour Technol 104:791–794. https://doi.org/10.1016/j.biortech.2011.10.017

76. Idler C, Venus J, Kamm B (2015) Microorganisms for the production of lactic acid and organic lactates. Microorg Biorefineries 26:225–273. https://doi.org/10.1007/978-3-662-45209-7_9
77. Bailly M (2002) Production of organic acids by bipolar electrodialysis: realizations and perspectives. Desalination 144(1–3):157–162. https://doi.org/10.1016/S0011-9164(02)00305-3
78. Choi JH, Kim SH, Moon SH (2002) Recovery of lactic acid from sodium lactate by ion substitution using ion-exchange membrane. Sep Purif Technol 28(1):69–79. https://doi.org/10.1016/S1383-5866(02)00014-X
79. Lee EG, Moon SH, Chang YK, Yoo IK, Chang HN (1998) Lactic acid recovery using two-stage electrodialysis and its modelling. J Membr Sci 145(1):53–66. https://doi.org/10.1016/S0376-7388(98)00065-9
80. Kim YH, Moon SH (2001) Lactic acid recovery from fermentation broth using one-stage electrodialysis. J Chem Technol Biotechnol 76(2):169–178. https://doi.org/10.1002/jctb.368
81. Martins PF, Medeiros HHR, Sbaite P, Maciel MW (2013) Enrichment of oxyterpenes from orange oil by short path evaporation. Sep Purif Technol 116:385–390. https://doi.org/10.1016/j.seppur.2013.06.011
82. Komesu A, Martins PF, Lunelli BH, Oliveira J, Maciel Filho R, Maciel MRW (2014) Evaluation of lactic acid purification from fermentation broth by hybrid short path evaporation using factorial experimental design. Sep Purif Technol 136:233–240. https://doi.org/10.1016/j.seppur.2014.09.010
83. Luongo V, Policastro G, Ghimire A, Pirozzi F, Fabbricino M (2019) Repeated-batch fermentation of cheese whey for semi-continuous lactic acid production using mixed cultures at uncontrolled pH. Sustainability 11(12):3330. https://doi.org/10.3390/su11123330
84. Kamble SP, Barve PP, Joshi JB, Rahman I, Kulkarni BD (2012) Purification of lactic acid via esterification of lactic acid using a packed column, followed by hydrolysis of methyl lactate using three continuously stirred tank reactors (CSTRs) in series: a continuous pilot plant study. Ind Eng Chem Res 51(4):1506–1514. https://doi.org/10.1021/ie200642j
85. Sanz MT, Murga R, Beltrán S, Cabezas JL, Coca J (2004) Kinetic study for the reactive system of lactic acid esterification with methanol: methyl lactate hydrolysis reaction. Ind Eng Chem Res 43(9):2049–2053. https://doi.org/10.1021/ie034031p
86. U.S. Patent 6,384,276,2002 Process for the preparation of lactic acid by evaporative crystallisation
87. Filachione EM, Fisher CH (1946) Purification of lactic acid. Ind Eng Chem 38(2):228–232
88. Arora MB, Hestekin JA, Snyder SW, St. Martin EJ, Lin YJ, Donnelly MI, Millard CS (2007) The separative bioreactor: a continuous separation process for the simultaneous production and direct capture of organic acids. Sep Sci Technol 42(11):2519–2538. https://doi.org/10.1080/01496390701477238
89. Bai DM, Jia MZ, Zhao XM, Ban R, Shen F, Li XG, Xu SM (2003) L (+)-lactic acid production by pellet-form *Rhizopus oryzae* R1021 in a stirred tank fermentor. Chem Eng Sci 58(3–6):785–791. https://doi.org/10.1016/S0009-2509(02)00608-5
90. Marták J, Schlosser Š, Sabolová E, Krištofíková L, Rosenberg M (2003) Fermentation of lactic acid with Rhizopus arrhizus in a stirred tank reactor with a periodical bleed and feed operation. Process Biochem 38(11):1573–1583. https://doi.org/10.1016/S0032-9592(03)00059-1
91. Park EY, Kosakai Y, Okabe M (1998) Efficient production of l-(+)-lactic acid using mycelial cotton-like flocs of *Rhizopus oryzae* in an air-lift bioreactor. Biotechnol Prog 14(5):699–704. https://doi.org/10.1021/bp9800642
92. Thongchul N, Yang ST (2003) Controlling filamentous fungal morphology by immobilization on a rotating fibrous matrix to enhance oxygen transfer and L (+)-lactic acid production by *Rhizopus oryzae*. ACS Symp Ser 862(3):36–51. https://doi.org/10.1021/bk-2003-0862.ch003
93. Du J, Cao N, Gong CS, Tsao GT (1998) Production of L-lactic acid by *Rhizopus oryzae* in a bubble column fermenter. Appl Biochem Biotechnol 70:323–329. https://doi.org/10.1007/BF02920148

94. Zhou Y, Domínguez JM, Cao N, Du J, & Tsao GT (1999) Optimization of L-lactic acid production from glucose by *Rhizopus oryzae* ATCC 52311. In Twentieth symposium on biotechnology for fuels and chemicals (pp. 401–407). Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-4612-1604-9_37
95. Miura S, Arimura T, Hoshino M, Kojima M, Dwiarti L, Okabe M (2003) Optimization and scale-up of l-lactic acid fermentation by mutant strain *Rhizopus* sp. MK-96-1196 in airlift bioreactors. J Biosci Bioeng 96(1):65–69. https://doi.org/10.1016/S1389-1723(03)90098-3
96. Yin P, Nishina N, Kosakai Y, Yahiro K, Pakr Y, Okabe M (1997) Enhanced production of L (+)-lactic acid from corn starch in a culture of *Rhizopus oryzae* using an air-lift bioreactor. J Ferment Bioeng 84(3):249–253. https://doi.org/10.1016/S0922-338X(97)82063-6
97. Silva EM, Yang ST (1995) Kinetics and stability of a fibrous-bed bioreactor for continuous production of lactic acid from unsupplemented acid whey. J Biotechnol 41(1):59–70. https://doi.org/10.1016/0168-1656(95)00059-Y
98. Eş I, Khaneghah AM, Barba FJ, Saraiva JA, Sant'Ana AS, Hashemi SMB (2018) Recent advancements in lactic acid production-a review. Food Res Int 107:763–770. https://doi.org/10.1016/j.foodres.2018.01.001
99. Shi Z, Wei P, Zhu X, Cai J, Huang L, Xu Z (2012) Efficient production of l-lactic acid from hydrolysate of Jerusalem artichoke with immobilized cells of *Lactococcus lactis* in fibrous bed bioreactors. Enzym Microb Technol 51(5):263–268. https://doi.org/10.1016/j.enzmictec.2012.07.007
100. Chotisubha-anandha N, Thitiprasert S, Tolieng V, Thongchul N (2011) Improved oxygen transfer and increased L-lactic acid production by morphology control of *Rhizopus oryzae* in a static bed bioreactor. Bioprocess Biosyst Eng 34(2):163–172. https://doi.org/10.1007/s00449-010-0457-z
101. Pimtong V, Ounaeb S, Thitiprasert S, Tolieng V, Sooksai S, Boonsombat R, Tanasupawat S, Assabumrungrat S, Thongchul N (2017) Enhanced effectiveness of *Rhizopus oryzae* by immobilization in a static bed fermentor for l-lactic acid production. Process Biochem 52:44–52. https://doi.org/10.1016/j.procbio.2016.09.020
102. Kosseva MR, Panesar PS, Kaur G, Kennedy JF (2009) Use of immobilised biocatalysts in the processing of cheese whey. Int J Biol Macromol 45(5):437–447. https://doi.org/10.1016/j.ijbiomac.2009.09.005
103. Faschian R, De S, Pörtner R (2016) Multi-fixed-bed bioreactor system applied for bioprocess development of immobilized lactic acid bacteria. Open Biotechnol J 10:1. https://doi.org/10.2174/1874070701610010001
104. Fan R, Ebrahimi M, Czermak P (2017) Anaerobic membrane bioreactor for continuous lactic acid fermentation. Membranes 7(2):26. https://doi.org/10.3390/membranes7020026
105. Smith Jr, CV (1969) The use of ultrafiltration membrane for activated sludge separation. In Proc. 24th Annual Purdue Industrial Waste Conf. (pp. 1300-1310)
106. Carrère H, Blaszkow F, de Balmann HR (2001) Modelling the clarification of lactic acid fermentation broths by cross-flow microfiltration. J Membr Sci 186(2):219–230. https://doi.org/10.1016/S0376-7388(00)00677-3
107. Cysewski GR, Wilke CR (1977) Rapid ethanol fermentations using vacuum and cell recycle. Biotechnol Bioeng 19(8):1125–1143. https://doi.org/10.1002/bit.260190804
108. Puziss M, Hedén CG (1965) Toxin production by clostridium tetani in biphasic liquid cultures. Biotechnol Bioeng 7(3):355–366. https://doi.org/10.1002/bit.260070305
109. Kwon S, Yoo IK, Lee WG, Chang HN, Chang YK (2001) High-rate continuous production of lactic acid by *lactobacillus rhamnosus* in a two-stage membrane cell-recycle bioreactor. Biotechnol Bioeng 73(1):25–34. https://doi.org/10.1002/1097-0290(20010405)73:1%3C25::AID-BIT1033%3E3.0.CO;2-N
110. Kulozik U, Hammelehle B, Pfeifer J, Kessler HG (1992) High reaction rate continuous bioconversion process in a tubular reactor with narrow residence time distributions for the production of lactic acid. J Biotechnol 22(1–2):107–116. https://doi.org/10.1016/0168-1656(92)90135-V

111. Jeantet R, Maubois JL, Boyaval P (1996) Semicontinuous production of lactic acid in a bioreactor coupled with nanofiltration membranes. Enzym Microb Technol 19(8):614–619. https://doi.org/10.1016/S0141-0229(96)00073-7
112. Wenten IG, Khoiruddin K, Aryanti PT, Victoria AV, Tanukusuma G (2018) Membrane-based zero-sludge palm oil mill plant. Rev Chem Eng 36(2):237–263. https://doi.org/10.1515/revce-2017-0117
113. Zhang Y, Chen X, Qi B, Luo J, Shen F, Su Y, Khan R, Wan Y (2014) Improving lactic acid productivity from wheat straw hydrolysates by membrane integrated repeated batch fermentation under non-sterilized conditions. Bioresour Technol 163:160–166. https://doi.org/10.1016/j.biortech.2014.04.038
114. Moueddeb H, Sanchez J, Bardot C, Fick M (1996) Membrane bioreactor for lactic acid production. J Membr Sci 114(1):59–71. https://doi.org/10.1016/0376-7388(95)00307-X
115. Tay A, Yang ST (2002) Production of L (+)-lactic acid from glucose and starch by immobilized cells of *Rhizopus oryzae* in a rotating fibrous bed bioreactor. Biotechnol Bioeng 80(1):1–12. https://doi.org/10.1002/bit.10340
116. Liu T, Miura S, Yaguchi M, Arimura T, Park EY, Okabe M (2006) Scale-up of L-lactic acid production by mutant strain *Rhizopus* sp. Mk-96-1196 from 0.003 m3 to 5 m3 in airlift bioreactors. J Biosci Bioeng 101(1):9–12. https://doi.org/10.1263/jbb.101.9
117. Abubakar A, Suberu HA, Bello IM, Abdulkadir R, Daudu OA, Lateef AA (2013) Effect of pH on mycelial growth and sporulation of *Aspergillus parasiticus*. J Plant Sci 1(4):64–67. https://doi.org/10.11648/j.jps.20130104.13
118. Niaz M, Iftikhar T, Qureshi FF, Niaz M (2014) Extracellular lipase production by *Aspergillus nidulans* (MBL-S-6) under submerged fermentation. Int J Agric Biol 16(3):536–542
119. Jin T, Zhang H (2008) Biodegradable polylactic acid polymer with nisin for use in antimicrobial food packaging. J Food Sci 73(3):M127–M134. https://doi.org/10.1111/j.1750-3841.2008.00681.x
120. Shahri SZ, Vahabzadeh F, Mogharei A (2020) Lactic acid production by loofah-immobilized *Rhizopus oryzae* through one-step fermentation process using starch substrate. Bioprocess Biosyst Eng 43(2):333–345. https://doi.org/10.1007/s00449-019-02231-5
121. Teke GM, Pott RW (2020) Design and evaluation of a continuous semipartition bioreactor for in situ liquid-liquid extractive fermentation. Biotechnol Bioeng 118(1):58–71. https://doi.org/10.1002/bit.27550
122. Singhvi M, Gokhale D (2013) Biomass to biodegradable polymer (PLA). RSC Adv 3(33):13558–13568. https://doi.org/10.1039/C3RA41592A
123. Farah S, Anderson DG, Langer R (2016) Physical and mechanical properties of PLA, and their functions in widespread applications—a comprehensive review. Adv Drug Deliv Rev 107:367–392. https://doi.org/10.1016/j.addr.2016.06.012
124. Gupta B, Revagade N, Hilborn J (2007) Poly (lactic acid) fiber: an overview. Prog Polym Sci 32(4):455–482. https://doi.org/10.1016/j.progpolymsci.2007.01.005
125. Jamshidian M, Tehrany EA, Imran M, Jacquot M, Desobry S (2010) Poly-lactic acid: production, applications, nanocomposites, and release studies. Compr Rev Food Sci Food Saf 9(5):552–571. https://doi.org/10.1111/j.1541-4337.2010.00126.x
126. Garlotta D (2001) A literature review of poly (lactic acid). J Polym Environ 9(2):63–84. https://doi.org/10.1023/A:1020200822435
127. Ghosh K, Jones BH (2021) Roadmap to biodegradable plastics—current state and research needs. ACS Sustain Chem Eng 9(18):6170–6187. https://doi.org/10.1021/acssuschemeng.1c00801
128. Emergen Research. Polylactic Acid Market By Product Type (Racemic PLLA (Poly-L-lactic Acid), Regular PLLA (Poly-L-lactic Acid), PDLA (Poly-D-lactic Acid), PDLLA (Poly-DL-lactic Acid)), Distribution Channel, BY Application (Packaging, Textile, Transport, Agriculture, Electronics, Medical), Forecasts to 2027. 2020, September [cited 2021; https://www.emergenresearch.com/industry-report/polylactic-acid-market

129. Mehta R, Kumar V, Bhunia H, Upadhyay SN (2005) Synthesis of poly (lactic acid): a review. J Macromol Sci Polym Rev 45(4):325–349. https://doi.org/10.1080/15321790500304148
130. Ahmed J, Varshney SK (2011) Polylactides—chemistry, properties and green packaging technology: a review. Int J Food Prop 14(1):37–58. https://doi.org/10.1080/10942910903125284
131. Lasprilla AJ, Martinez GA, Lunelli BH, Jardini AL, Maciel Filho R (2012) Poly-lactic acid synthesis for application in biomedical devices—a review. Biotechnol Adv 30(1):321–328. https://doi.org/10.1016/j.biotechadv.2011.06.019
132. Van Hilst F, Hoefnagels R, Junginger M, Shen L, Wicke B (2017) Sustainable biomass for energy and materials: a greenhouse gas emission perspective. Copernicus Institute of Sustainable Development Working Paper, Utrecht
133. Masutani K, & Kimura Y (2014) PLA synthesis. From the monomer to the polymer https://doi.org/10.1039/9781782624806-00001
134. Ehsani M, Khodabakhshi K, Asgari M (2014) Lactide synthesis optimization: investigation of the temperature, catalyst and pressure effects. E-Polymers 14(5):353–361. https://doi.org/10.1515/epoly-2014-0055
135. Moon SI, Lee CW, Taniguchi I, Miyamoto M, Kimura Y (2001) Melt/solid polycondensation of L-lactic acid: an alternative route to poly (L-lactic acid) with high molecular weight. Polymer 42(11):5059–5062. https://doi.org/10.1016/S0032-3861(00)00889-2
136. Mathew AP, Oksman K, Sain M (2005) Mechanical properties of biodegradable composites from poly lactic acid (PLA) and microcrystalline cellulose (MCC). J Appl Polym Sci 97(5):2014–2025. https://doi.org/10.1002/app.21779
137. Nagahata R, Sano D, Suzuki H, Takeuchi K (2007) Microwave-assisted single-step synthesis of poly (lactic acid) by direct polycondensation of lactic acid. Macromol Rapid Commun 28(4):437–442. https://doi.org/10.1002/marc.200600715
138. Cao HL, Wang P, Yuan WB (2009) Microwave-assisted synthesis of poly (L-lactic acid) via direct melt polycondensation using solid super-acids. Macromol Chem Phys 210(23):2058–2062. https://doi.org/10.1002/macp.200900231
139. Zahari SSNS, Mansor MH, Azman HH, Rosli D (2020) Uncatalysed Polycondensation of Lactic Acid to Polylactic Acid under Microwave Irradiation: Effect of Microwave Power. J Phys: Conference Series 1551(1):012001). IOP Publishing. https://doi.org/10.1088/1742-6596/1551/1/012001
140. Li G, Zhao M, Xu F, Yang B, Li X, Meng X, Teng L, Sun F, Li Y (2020) Synthesis and biological application of polylactic acid. Molecules 25(21):5023. https://doi.org/10.3390/molecules25215023
141. Kim KW, Woo SI (2002) Synthesis of high-molecular-weight poly (L-lactic acid) by direct polycondensation. Macromol Chem Phys 203(15):2245–2250. https://doi.org/10.1002/1521-3935(200211)203:15<2245::AID-MACP2245>3.0.co;2-3
142. Hadiantoro S, Widjajanti K, Putri DIK, & Nikmah L (2021) Poly lactic acid (pla) development using lactic acid product from rice straw fermentation with azeotropic polycondensation process. In IOP Conference Series: Materials Science and Engineering (Vol. 1053, No. 1, p. 012043). IOP Publishing. https://doi.org/10.1088/1757-899x/1053/1/012043
143. Avérous L (2008) Polylactic acid: synthesis, properties and applications. In Monomers, polymers and composites from renewable resources (pp. 433–450). Elsevier. https://doi.org/10.1016/b978-0-08-045316-3.00021-1
144. Kricheldorf HR, Dunsing R (1986) Polylactones, 8. Mechanism of the cationic polymerization of L, L-dilactide. Die Makromolekulare Chemie. Macromol Chem Phys 187(7):1611–1625. https://doi.org/10.1002/macp.1986.021870706
145. Baśko M, Kubisa P (2010) Cationic polymerization of l, l-lactide. J Polym Sci A Polym Chem 48(12):2650–2658. https://doi.org/10.1002/pola.24048
146. Penczek S (1980) Cationic ring-opening polymerization of heterocyclic monomers. I Mechanisms https://doi.org/10.1007/bfb0023043

147. Sutar AK, Maharana T, Dutta S, Chen CT, Lin CC (2010) Ring-opening polymerization by lithium catalysts: an overview. Chem Soc Rev 39(5):1724–1746. https://doi.org/10.1039/b912806a
148. Uyama H, & Kobayashi S (2019) Synthesis of polyesters II: hydrolase as catalyst for ring-opening polymerization. Enzymatic polymerization towards green polymer chemistry: green chemistry and sustainable technology; Kobayashi, S., Uyama, H., Kadokawa, J., eds, 165-197. https://doi.org/10.1007/978-981-13-3813-7_6
149. Zhao H, Nathaniel GA, Merenini PC (2017) Enzymatic ring-opening polymerization (ROP) of lactides and lactone in ionic liquids and organic solvents: digging the controlling factors. RSC Adv 7(77):48639–48648. https://doi.org/10.1039/c7ra09038b
150. Whulanza Y, Rahman SF, Suyono EA, Yohda M, Gozan M (2018) Use of Candida rugosa lipase as a biocatalyst for L-lactide ring-opening polymerization and polylactic acid production. Biocatal Agric Biotechnol 16:683–691. https://doi.org/10.1016/j.bcab.2018.09.015
151. Bukhari A, Atta M, Idris A (2017) Synthesis of poly (L-) lactide under simultaneous cooling and microwave heating by using immobilised Candida Antarctica lipase B. Chem Eng Trans 56:1447–1452. https://doi.org/10.3303/CET1756242
152. Auras R, Harte B, Selke S (2004) An overview of polylactides as packaging materials. Macromol Biosci 4(9):835–864. https://doi.org/10.1002/mabi.200400043
153. Eerikäinen T, Linko P, Linko S, Siimes T, Zhu YH (1993) Fuzzy logic and neural network applications in food science and technology. Trends Food Sci Technol 4(8):237–242. https://doi.org/10.1016/0924-2244(93)90137-Y
154. Mohammadi V, & Minaei S (2019) Artificial intelligence in the production process. In Engineering tools in the beverage industry (pp. 27–63). Woodhead Publishing. https://doi.org/10.1016/B978-0-12-815258-4.00002-0
155. Singh A, Majumder A, Goyal A (2008) Artificial intelligence based optimization of exocellular glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146. Bioresour Technol 99(17):8201–8206. https://doi.org/10.1016/j.biortech.2008.03.038
156. Ng A (2000) CS229 Lecture notes CS229 Lecture notes, 1(1), 1–3. https://backspaces.net/temp/ML/CS229.pdf
157. Karray F, Karray FO, De Silva CW (2004) Soft computing and intelligent systems design: theory, tools, and applications. Pearson Education
158. Chaturvedi DK (2008) Artificial neural network and supervised learning. Soft Computing: Techniques and its Applications in Electrical Engineering, 23-50. https://doi.org/10.1007/978-3-540-77481-5_3
159. Panagou EZ, Kodogiannis V, Nychas GE (2007) Modelling fungal growth using radial basis function neural networks: the case of the ascomycetous fungus *Monascus ruber* van *Tieghem*. Int J Food Microbiol 117(3):276–286. https://doi.org/10.1016/j.ijfoodmicro.2007.03.010
160. Ahmad A, Banat F, Taher H (2021) Comparative study of lactic acid production from date pulp waste by batch and cyclic–mode dark fermentation. Waste Manag 120:585–593. https://doi.org/10.1016/j.wasman.2020.10.029
161. Lunelli BH, Melo DN, de Morais ER, Victorino IR, de Toledo ECV, Maciel MRW, & Maciel Filho R (2011) Real-time optimization for lactic acid production from sucrose fermentation by *Lactobacillus plantarum*. In Computer aided chemical engineering (vol. 29, pp. 1396-1400). Elsevier. https://doi.org/10.1016/B978-0-444-54298-4.50058-1
162. Carroll DL (1996) Chemical laser modeling with genetic algorithms. AIAA J 34(2):338–346. https://doi.org/10.2514/3.13069
163. Dulf EH, Vodnar DC, Dulf FV (2018) Modeling tool using neural networks for l (+)-lactic acid production by pellet-form *Rhizopus oryzae* NRRL 395 on biodiesel crude glycerol. Chem Cent J 12(1):1–9. https://doi.org/10.1186/s13065-018-0491-5
164. Saleh B, Maher I, Abdelrhman Y, Heshmat M, Abdelaal O (2021) Adaptive neuro-fuzzy inference system for modelling the effect of slurry impacts on PLA material processed by FDM. Polymers 13(1):118. https://doi.org/10.3390/polym13010118

165. Jakkula V (2006) Tutorial on support vector machine (svm). School of EECS, Washington State University, 37. https://course.ccs.neu.edu/cs5100f11/resources/jakkula.pdf
166. Segler MH, Preuss M, Waller MP (2018) Planning chemical syntheses with deep neural networks and symbolic AI. Nature 555(7698):604–610. https://doi.org/10.1038/nature25978
167. Singhvi MS, Zinjarde SS, Gokhale DV (2019) Polylactic acid: synthesis and biomedical applications. J Appl Microbiol 127(6):1612–1626. https://doi.org/10.1111/jam.14290
168. Yanoso-Scholl L, Jacobson JA, Bradica G, Lerner AL, O'Keefe RJ, Schwarz EM, Zuscik MJ, Awad HA (2010) Evaluation of dense polylactic acid/beta-tricalcium phosphate scaffolds for bone tissue engineering. J Biomed Mater Res A 95(3):717–726. https://doi.org/10.1002/jbm.a.32868
169. Haaparanta AM, Järvinen E, Cengiz IF, Ellä V, Kokkonen HT, Kiviranta I, Kellomäki M (2014) Preparation and characterization of collagen/PLA, chitosan/PLA, and collagen/chitosan/PLA hybrid scaffolds for cartilage tissue engineering. J Mater Sci Mater Med 25(4):1129–1136. https://doi.org/10.1007/s10856-013-5129-5
170. Gupta S, Sharma A, Kumar JV, Sharma V, Gupta PK, Verma RS (2020) Meniscal tissue engineering via 3D printed PLA monolith with carbohydrate based self-healing interpenetrating network hydrogel. Int J Biol Macromol 162:1358–1371. https://doi.org/10.1016/j.ijbiomac.2020.07.238
171. Mohandesnezhad S, Pilehvar-Soltanahmadi Y, Alizadeh E, Goodarzi A, Davaran S, Khatamian M, Zarghami N, Samiei M, Aghazadeh M, Akbarzadeh A (2020) In vitro evaluation of Zeolite-nHA blended PCL/PLA nanofibers for dental tissue engineering. Mater Chem Phys 252:123152. https://doi.org/10.1016/j.matchemphys.2020.123152
172. Dong Y, Feng SS (2006) Nanoparticles of poly (D, L-lactide)/methoxy poly (ethylene glycol)-poly (D, L-lactide) blends for controlled release of paclitaxel. J Biomed Mater Res Part A 78(1):12–19. https://doi.org/10.1002/jbm.a.30684
173. Siafaka PI, Barmbalexis P, Bikiaris DN (2016) Novel electrospun nanofibrous matrices prepared from poly (lactic acid)/poly (butylene adipate) blends for controlled release formulations of an anti-rheumatoid agent. Eur J Pharm Sci 88:12–25. https://doi.org/10.1016/j.ejps.2016.03.021
174. Yu Y, Chen CK, Law WC, Weinheimer E, Sengupta S, Prasad PN, Cheng C (2014) Polylactide-graft-doxorubicin nanoparticles with precisely controlled drug loading for pH-triggered drug delivery. Biomacromolecules 15(2):524–532. https://doi.org/10.1021/bm401471p
175. Mhlanga N, Sinha Ray S, Lemmer Y, Wesley-Smith J (2015) Polylactide-based magnetic spheres as efficient carriers for anticancer drug delivery. ACS Appl Mater Interfaces 7(40):22692–22701. https://doi.org/10.1021/acsami.5b07567
176. Li W, Fan X, Wang X, Shang X, Wang Q, Lin J, Hu Z, Li Z (2018) Stereocomplexed micelle formation through enantiomeric PLA-based Y-shaped copolymer for targeted drug delivery. Mater Sci Eng C 91:688–695. https://doi.org/10.1016/j.msec.2018.06.006
177. Chen J, Tian B, Yin X, Zhang Y, Hu D, Hu Z, Liu M, Pan Y, Zhao J, Li H, Hou C, Wang J, Zhang Y (2007) Preparation, characterization and transfection efficiency of cationic PEGylated PLA nanoparticles as gene delivery systems. J Biotechnol 130(2):107–113. https://doi.org/10.1016/j.jbiotec.2007.02.007
178. Camović M, Biščević A, Brčić I, Borčak K, Bušatlić S, Ćenanović N, Dedović A, Mulalić A, Osmanlić M, Sirbubalo M, Tucak A, & Vranić E (2019) Coated 3d printed PLA microneedles as transdermal drug delivery systems. In International conference on medical and biological engineering (pp. 735–742). Springer, Cham. https://doi.org/10.1007/978-3-030-17971-7_109
179. Vaňková E, Kašparová P, Khun J, Machková A, Julák J, Sláma M, Hodek J, Ulrychová L, Weber J, Obrová K, Kosulin K, Lion T, Scholtz V (2020) Polylactic acid as a suitable material for 3D printing of protective masks in times of COVID-19 pandemic. PeerJ 8:e10259. https://doi.org/10.7717/peerj.10259

180. Buluş E, Buluş GS, Yakuphanoglu F (2020) Production of polylactic acid-activated charcoal nanofiber membranes for COVID-19 pandemic by electrospinning technique and determination of filtration efficiency. J Mater Electronic Devices 4(1):21–26
181. Alghounaim M, Almazeedi S, Al Youha S, Papenburg J, Alowaish O, AbdulHussain G, Rawan Al-Shemali R, Albuloushi A, Alzabin S, Al-Wogayan K, Al-Mutawa Y, Al-Sabah S (2020) Low-cost polyester-tipped three-dimensionally printed nasopharyngeal swab for the detection of severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2). J Clin Microbiol 58(11):e01668–20. https://doi.org/10.1128/jcm.01668-20
182. Holm VK, Mortensen G, Vishart M, Petersen MA (2006) Impact of poly-lactic acid packaging material on semi-hard cheese. Int Dairy J 16(8):931–939. https://doi.org/10.1016/j.idairyj.2005.09.001
183. Lukic I, Vulic J, Ivanovic J (2020) Antioxidant activity of PLA/PCL films loaded with thymol and/or carvacrol using scCO2 for active food packaging. Food Packag Shelf Life 26:100578. https://doi.org/10.1016/j.fpsl.2020.100578
184. Moustafa H, El Kissi N, Abou-Kandil AI, Abdel-Aziz MS, Dufresne A (2017) PLA/PBAT bionanocomposites with antimicrobial natural rosin for green packaging. ACS Appl Mater Interfaces 9(23):20132–20141. https://doi.org/10.1021/acsami.7b05557
185. Knoch S, Pelletier F, Larose M, Chouinard G, Dumont MJ, Tavares JR (2020) Surface modification of PLA nets intended for agricultural applications. Colloids Surf A Physicochem Eng Asp 598:124787. https://doi.org/10.1016/j.colsurfa.2020.124787
186. Khan H, Kaur S, Baldwin TC, Radecka I, Jiang G, Bretz I, Duale K, Adamus G, & Kowalczuk M (2020) Effective control against broadleaf weed species provided by biodegradable PBAT/PLA mulch film embedded with the herbicide 2-methyl-4-chlorophenoxyacetic acid (MCPA). https://doi.org/10.1021/acssuschemeng.0c00991
187. Souza PMS, Sommaggio LRD, Marin-Morales MA, Morales AR (2020) PBAT biodegradable mulch films: study of ecotoxicological impacts using *Allium cepa*, *Lactuca sativa* and HepG2/C3A cell culture. Chemosphere 256:126985. https://doi.org/10.1016/j.chemosphere.2020.126985
188. Jabbar A, Tausif M, Tahir HR, Basit A, Bhatti MRA, Abbas G (2019) Polylactic acid/lyocell fibre as an eco-friendly alternative to polyethylene terephthalate/cotton fibre blended yarns and knitted fabrics. J Text Inst 111(1):129–138. https://doi.org/10.1080/00405000.2019.1624070
189. Huang XX, Tao XM, Zhang ZH, Chen P (2016) Properties and performances of fabrics made from bio-based and degradable polylactide acid/poly (hydroxybutyrate-co-hydroxyvalerate) (PLA/PHBV) filament yarns. Text Res J 87(20):2464–2474. https://doi.org/10.1177/0040517516671128

Discovery of Bioactive Metabolites from the Genus *Arthrinium*

9

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Abstract

The ascomycete genus *Arthrinium* (class, Sordariomycetes; family, Apiosporaceae) comprises approximately 88 species distributed worldwide. It has been reported as saprobes, plant pathogens and endophytes worldwide. It has been isolated from different terrestrial environments (e.g. soil, air, plants and marine ecosystems). *Arthrinium* is known to produce compounds like arthrichitin and arundifungin. Various classes of chemical compounds are produced by *Arthrinium* spp., including alkaloids, peptides, indoles, terpenes, terpenoid, coumarin, xanthone, pyrone and naphthol. Secondary metabolites derived from *Arthrinium* spp. display an extensive biological potential: antibacterial, antifungal, anti-inflammatory, anticancer and antioxidants. The purpose of this review is to follow the metabolites reported from the genus *Arthrinium* and their biological activities.

Keywords

Arthrinium species · Secondary metabolites · Anticancer compounds · Antimicrobial compounds · Arthrichitin · Arundifungin · Antioxidants · Wormicides · Biotransformation

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9.1 Introduction

The genus *Arthrinium* (division, Ascomycota; class, Sordariomycetes; family, Apiosporaceae) was first recorded over 200 years ago, with *Arthrinium caricicola* as a type species [1]. To date, this genus comprises of about 88 species distributed globally (Index Fungorum: http://www.indexfungorum.org). *Arthrinium* spp. were reported as saprobes, plant pathogens and endophytes worldwide. They are the inhabitants of terrestrial habitats (e.g. soil, air and plants) [2–4]. *Arthrinium* spp. are also inhabitants of marine environments (e.g. seawater, seaweed and sponges) [5–12]. Besides marine sponges and seaweed, they are also reported from the egg masses of sailfin sandfish [5, 8, 13]. Many *Arthrinium* spp. (e.g. *A. arundinis*, *A. phaeospermum*, *A. rasikravindrae*, *A. sacchari* and *A. saccharicola*) are the inhabitants of terrestrial as well as marine environments [4]. The genus *Arthrinium* is well-known to produce compounds like arthrichitin and arundifungin. Various groups of chemical compounds are produced by various *Arthrinium* species, including alkaloids, peptides, indoles, terpenes, terpenoid, coumarin, xanthone, pyrone and naphthol. Secondary metabolites derived from *Arthrinium* spp. display a varied range of bioactive compounds with anticancer [14, 15], anti-inflammatory [16], antioxidant [17, 18], antibacterial [12, 19] and antifungal [20, 21] activities. The present review intends to highlight the bioactive metabolites reported from the genus *Arthrinium* and their biological activities. The specifications of bioactive compounds isolated from *Arthrinium* spp. with their biological potential are presented in Table 9.1.

9.2 Bioactive Potential from *Arthrinium*

The genus *Arthrinium* produces a large number of bioactive metabolites with various biological activities. Some of them have been reported here.

9.2.1 Antifungal Compounds

From *Arthrinium phaeospermum* (HIL Y-903022) occurring as an endophyte in an unidentified grass collected from Mulund (Mumbai, India), a novel cell wall active depsipeptide arthrichitin **(1)** (Fig. 9.1) has been isolated. Its structural features have been elucidated based on spectroscopic as well as chemical degradation studies. Serine, â-keto tryptophan, glutamic acid and 2,4-dimethyl-3-hydroxydodecanoic acid are all found in arthrichitin. Arthrichitin **(1)** exhibited poor antifungal activity against *Candida* sp., *Trichophyton* sp. and many plant pathogens. In vitro, arthrichitin **(1)** has caused morphological abnormalities in *Botrytis cinerea*. It has a fungicidal effect between 75% and 85% against infection caused by *Pyricularia oryzae* in rice and *B. cinerea* in cucumbers under greenhouse conditions at 5000 ppm, respectively. It is also similar to nikkomycin Z, which binds as well as inhibits chitin synthases (Chs1 and Chs2) in *C. albicans*. Since it has poor in vitro

Table 9.1 Novel bioactive compounds reported from the genus *Arthrinium*

Sr. No.	Producing organism	Name of the compound	Biological target	Biological activity[a] (MIC/IC_{50}/ID_{50})	References
Antifungal activity					
1.	*Arthrinium phaeospermum*	Arthrichitin **(1)**	*Candida* sp., *Trichophyton* sp.	Weak activity	[21]
			Botrytis cinerea		
			Fungicidal efficiency against infection of *Pyricularia oryzae* to rice and *B. cinerea* to cucumber	75% and 85% at 5000 ppm	
2.	*Arthrinium arundinis*	Arundifungin **(2)**	Active against number of *Candida* spp. and *Aspergillus fumigatus*	MIC in the range of 2 to 8 μg/ml and 1 μg/ml and morphological abnormalities in vitro	[20]
3.	*A. phaeospermum*	Arthrinic acid **(3)**	*Botrytis cinerea*, *Rhizopus stolonifera* and *Diplodia pinea*	MIC approx. 50 μg/ml	[22]
4.	*Arthrinium arundinis* TE-3	Diorcinol M–O **(4–6),** compounds **(7–9)**			[23]
		Compounds **(4)** and **(5)**	*Mucor hiemalis*	MIC of 8 and 4 μg/mL	
		Compounds **(8)** and **(9)**	*Alternaria alternata*	MIC of 8 μg/mL	
Anticancer activity					
5.	*Arthrinium* sp.	(−)-Hexylitaconic acid **(10)**	Inhibited the p53-HDM2 binding	IC_{50} 50 μg/mL	[11]
6.	*Arthrinium* sp.	Arthrinins A–D **(11–14)** and myrocin D **(15),** myrocin A **(16)**, norlichexanthone **(17)**, anomalin A **(18)**, decarboxycitrinone **(19)**			[14]

(continued)

Table 9.1 (continued)

Sr. No.	Producing organism	Name of the compound	Biological target	Biological activity[a] (MIC/IC_{50}/ID_{50})	References
		and 2,5-dimethyl-7-hydroxychromone **(20)**			
		Compound **(17)**	L5178Y, K562, A2780 and A2780 CisR cell lines	IC_{50} values of 1.16, 253.50, 68.2 and 74.0 μM	
		Compound **(18)**	L5178Y, cell lines	IC_{50} values of 0.40 μM	
		Compounds **(17)** and **(18)**	Protein kinases, viz. aurora-B, PIM1 and VEGF-R2	IC_{50} values in the range of 0.3 to 11.7 μM	
		Compounds **(18, 15** and **16)**	VEGF-A-dependent endothelial cell sprouting	IC_{50} values of 1.8, 2.6 and 3.7 μM	
7.	*Arthrinium sacchari*	Myrocin D **(15)**, libertellenone E **(21)**, libertellenone F **(22)**, decarboxyhydroxycitrinone **(23)**, myrocin A **(16)** and libertellenone C **(24)**, cytochalasin E **(25)**			[24]
		Compounds **(16, 23–24)**	HUVECs and HUAECs	IC_{50} values ranging from 7.6 to 68.7μM.	
		Compound **(25)**	HUVECs and HUAECs	IC_{50}, 0.0114 and 0.0110 μM	
		Positive control Ki8751	HUVECs and HUAECs	IC_{50}, 1.0–2.0 μM	
8.	*Arthrinium arundinis* ZSDS1-F3	Arthriniumnins A–D **(26–29)**, ketocytochalasin **(30)**, cytochalasin K **(31)**, cytochalasin Z16 **(32)**, rosellichalasin **(33)**, 10-phenyl-[12]-cytochalasin Z16 **(34)** and cytochalasin Z17 **(35)**			[25]
		Compounds **(31)**, **(34)**	K562, A549, Huh-7, H1975, MCF-7, U937, BGC823, HL60, Hela and MOLT-4 cell lines	IC_{50} values ranging from 1.13 to 47.4 μM	

9.	*Arthrinium* sp. A092	Arthrinisocoumarin A **(36)**, decarboxycitrinone **(19)**, 4-hydroxy-17R-methylincisterol **(37)**, 4-hydroxy-3-methoxybenzoic acid **(38)**, dibutyl phthalate **(39)**, flemingipanic acid **(40)**, indole-3-carboxy acid **(41)**, ergosterol peroxide **(42)**, p-hydroxybenzoic acid **(43)** and 4-hydroxybenzaldehyde **(44)**			[26]
		Compounds **(37)**	SF-268 and MCF-7	IC_{50} values of 63.8 and 57.2 μM	
		Compounds **(42)**	SF-268, MCF-7 and NCI-H460	IC_{50} values of 50.6, 32.3 and 39.0 μM	
10.	*A. arundinis* ZSDS1-F3	5-Hydroxy-4-oxohexanoate **(45)**, trans-(3R,4S)-(−)-4-hydroxymellein **(46)**, cis-(3R,4R)-(−)-4-hydroxymellein **(47)**, R-(−)-5-hydroxymellein **(48)**, decarboxycitrinone **(19)** and altechromone A **(49)**			[8]
		Compounds **(46)** and **(47)**	HeLa, DU145, U937 and HL60 cell lines	IC_{50} values ranging from 3.8 to 41.1 μM	
11.	*Arthrinium* sp.	Spiroarthrinols A **(50)** and B **(51),** griseofulvin **(52)** and its dechloroderivative **(53)**, 7-hydroxy-2-(2-hydroxypropyl)-5-methylchromone **(54)**, emodin **(55)**, chrysophanol **(56)**, endocrocin **(57)**, 3,8-dihydroxy-6-methoxy-8-methylxanthone	Caco-2 cell lines	No significant cytotoxicity	[5]

(continued)

Table 9.1 (continued)

Sr. No.	Producing organism	Name of the compound	Biological target	Biological activity[a] (MIC/IC_{50}/ID_{50})	References
		(58), norlichexanthone **(59)**, 2 (4-hydroxyphenyl)ethylacetate **(60),** 2-(4-hydroxyethyl) phenol (tyrosol) **(61)**, phomonitroester **(62)**, 3-nitropropionic acid **(63)**, 3,4-dimethoxybenzoic acid **(64)**, 1:1 mixture of 3-phenylpropane-1,2-diol **(65),** 4-hydroxyphenylacetic acid **(66)**, 8-O-4 dehydrodiferulic acid **(67)**, 3β,22-dihydroxylanosta-7,9 (11),24-triene **(68)** and dankasterone A **(69)**			
12.	*A. arundinis* TE-3	Compounds **(5**, **8** and **9)**	THP-1 cell line	IC_{50}, 40.2, 28.3 and 25.9 μM	[23]
13.	*A. arundinis* TE-3	Diorcinol N **(5)**	A3 cells	IC_{50} value of 16.31 μM	[15]
14.	*Arthrinium* sp. UJNMF0008	Arthpyrones D–K **(70–77)**, apiosporamide **(78)** and arthpyrone B **(79)**			[33]
		Apiosporamide **(78)**	U2OS and MG63	IC_{50} values of 19.3 and 11.7 μM	
15.	*Arthrinium* sp. (5XNZ5–4)	Terpestacin B **(80)**, 21-hydroxyterpestacin **(81),** terpestacin **(82)**		No activity in antibacterial and cytotoxicity assays	[27]
16.	*Arthrinium* sp.	Gentisyl alcohol **(83)**	MAPK and PI3K/AKT pathways	Upregulated signal transduction	[28]
17.	*Arthrinium* sp. NF2410	Arthrianhydride A **(84)** and B **(85)**, Compounds **(86–88)**			[29]
		Compounds **(84** and **85)**	SHP2	Inhibitory rate of 63.02 and 12.80% at 30 μM concentration	

18.	*Arthrinium marii* M-211	(6R,7R,8R)-theissenone A **(89)**, (6S,7R,8R)-theissenone **(90)** and arthrinone **(91)**	H4IIE rat hepatoma cells	IC_{50} values of 67.5, 46.6 and 13.4 μM	[30]
19.	*Arthrinium* sp. UJNMF0008	Arthpyrone L **(92)**	MG63, U2OS, A549, MCF-7 and MDA-MB-231 cell	IC_{50} of 7.14, 13.34, 17.56, 22.33 and 24.05 μM	[31]
		Positive control doxorubicin		IC_{50} of 3.56, 11.17, 2.40, 1.94 and 2.93 nM	
20.	*Arthrinium* sp.	2,3,4,6,8-pentahydroxy-1-methylxanthone **(93)** and arthone C **(94)**		Inhibited ox-LDL-induced apoptosis and adhesion factor expression in HUVECs. Protected endothelial cells against ox-LDL-induced oxidative stress through activating the AKT/Nrf2/HO-1 pathway	[32]
21.	*Arthrinium arundinis* DJ-13	Arundisins A–F **(95–100)**, Compounds **(101–111)**, ergosterol peroxide **(42)**,			[19].
		arundisins A **(95)** and B **(96)**	MCF-7	IC_{50}, 18.82 and 15.20 μM	
Antibacterial activity					
22.	*Arthrinium* sp. UJNMF0008	Arthpyrones F–I **(72–75)**, apiosporamide **(78)**	*Mycobacterium smegmatis* and *Staphylococcus aureus*	IC_{50} ranging from 1.66 to 42.8 μM	[33]
23.	*Arthrinium arundinis* DJ-13	Arundisin F **(100)**, Arundisin D **(98)**	*Escherichia coli*, *Candida albicans*	MIC of 8.00 μg/mL MIC of 32.00 μg/mL	[19]
24.	*Arthrinium* sp.	(+)-5-Chlorogriseofulvin **(112)**, griseofulvin **(113)** and dechlorogriseofulvin **(114)**,	*Micrococcus luteus*	MIC of 100 μM	[12]
		Compound **(112)**	Brine shrimp *Artemia salina*	LC_{50} value of 25 μg/mL	
Anti-inflammatory activity					
25.	*Arthrinium* sp. ZSDS1-F3	1,8-Dihydroxynaphthol-1-O-α-l-rhamnopyranoside **(115)**,			[16]

(continued)

Table 9.1 (continued)

Sr. No.	Producing organism	Name of the compound	Biological target	Biological activity[a] (MIC/IC_{50}/ID_{50})	References
		alternariol **(116)** and five known xanthones, 1,3,6-trihydroxy-8-methylxanthone **(117)**, 1,3,5,6-tetrahydroxy-8-methylxanthone **(118)**, anomalins A **(119)** and B **(120)**, fischexanthone **(121)**,			
		Compound **(117)**	COX-2 inhibition	IC_{50} value of 12.2 μM	
Antioxidant activity					
26.	*Arthrinium* sp. UJNMF0008	Arthones A–E **(122–126)**, AGI-B4 **(127)**, 1,3,6-trihydroxy-8-methylxanthone **(120)**, 2,3,4,6,8-pentahydroxy-1-methylxanthone **(128)**, sydowinin A **(129)**, sydowinin B **(130)**, conioxanthone A **(131)**, engyodontiumone B **(132)** and 8-hydroxy-3-hydroxymethyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ester **(133)**			[17]
		Compounds **(124)** and **(128)**	DPPH and ABTS radical scavenging assay	IC_{50} values ranging from 16.9 to 18.7 μM	
27.	*Arthrinium* sp.	Gentisyl alcohol **(83)**	DPPH and ABTS assay	IC_{50} 28.74 and 26.43 μM	[18]
		Positive control ascorbic acid		IC_{50}, 38.6 and 77.79 μM	
Wormicidal activity					
28.	*Arthrinium* state of *Apiospora montagnei*	R-(−)-mellein **(134)** and cis-(3R,4R)-4-hydroxymellein **(135)**	*Schistosoma mansoni* adult worms	Caused death of 100% of at 200 and 50 μg /mL	[34]

Some other compounds from Arthrinium					
29.	*A. arundinis* ZSDS1-F3	Arthpyrones A–C **(136–138)**, N-hydroxyapiosporamide **(139)**			[35]
30.	*Arthrinium phaeospermum* (JS 0567)	2, 3, 6, 8-Tetrahydroxy-1-methylxanthone (**140**), 2, 3, 4, 6, 8- pentahydroxy-1-methylxanthone (**141**), 3, 4, 6, 8-tetrahydroxy-1-methylxanthone (**142**), 3, 6, 8-trihydroxy-1-methylxanthone (**143**), 2, 4,2′,4′,6′-pentahydroxy-6-methylbenzophenone (**144**) and 5,7-di hydroxy-3-methylphthalide (**145**)			[48]
31.	*Arthrinium* sp. (JS420)	Tyrosol **(61)**, (3R,4R)-(−)-4-hydroxymellein **(146)**, (3R,4S)-(−)-4-hydroxymellein **(147)** and 1-phenyl-1,2-ethanediol **(148)**			[36]
32.	*Arthrinium arundinis*	2,8-Dihydroxy-9-oxo-9H-xanthene-6-carboxylic acid **(149)**, 2,8-dihydroxy-1-methoxycarbonyl-9-oxo-9H-xanthene-6-carboxylic acid **(150)**, 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether **(151)**, 1,3,6-trihydroxy-8-methylxanthone **(120)**, 1,6-dihydroxy-3-methoxy-8-methylxanthone **(152)**,			[37]

(continued)

Table 9.1 (continued)

Sr. No.	Producing organism	Name of the compound	Biological target	Biological activity[a] (MIC/IC_{50}/ID_{50})	References
		decarboxycitrinone **(19)** and formoic acid A **(153)**			
33.	*Arthrinium sacchari*	Saccharomone A **(154)**, 8-hydroxy-3-methyl-9-oxo9H-xanthene-1-carboxylic acid methyl ester **(155)**, engyodontiumone H **(156)**, AGI-B4 **(157),** 2-hexyl-3-methylmaleic anhydride **(158)** and bostrycin **(159)**			[38]
34.	*Arthrinium phaeospermum*	Bostrycin **(159)**			[39]
35.	*Arthrinium* sp. HS66	Arthrinins E–G **(160–162)**			[40]
36.	*Arthrinium* sp.	Arthrinic acid derivatives **(163,164)**, one phenolic derivative **(165)** and (S)-3-hydroxy-6-(2-hydroxypropyl)-5-methyl-2H-pyran-2-one **(166)** and methyl ester of arthrinic acid **(167)**			[41]
37.	*Shiraia bambusicola* co-cultured with *Arthrinium* sp. *AF-5*	Hypocrellin A **(168)**	Yield improved, four times of that by the fermentation only with the *S. bambusicola*		[42]
Biotransformation					
38.	*Arthrinium* sp. GE 17–18	Biotransform ginsenoside Rb1 to ginsenoside C–K			[43]

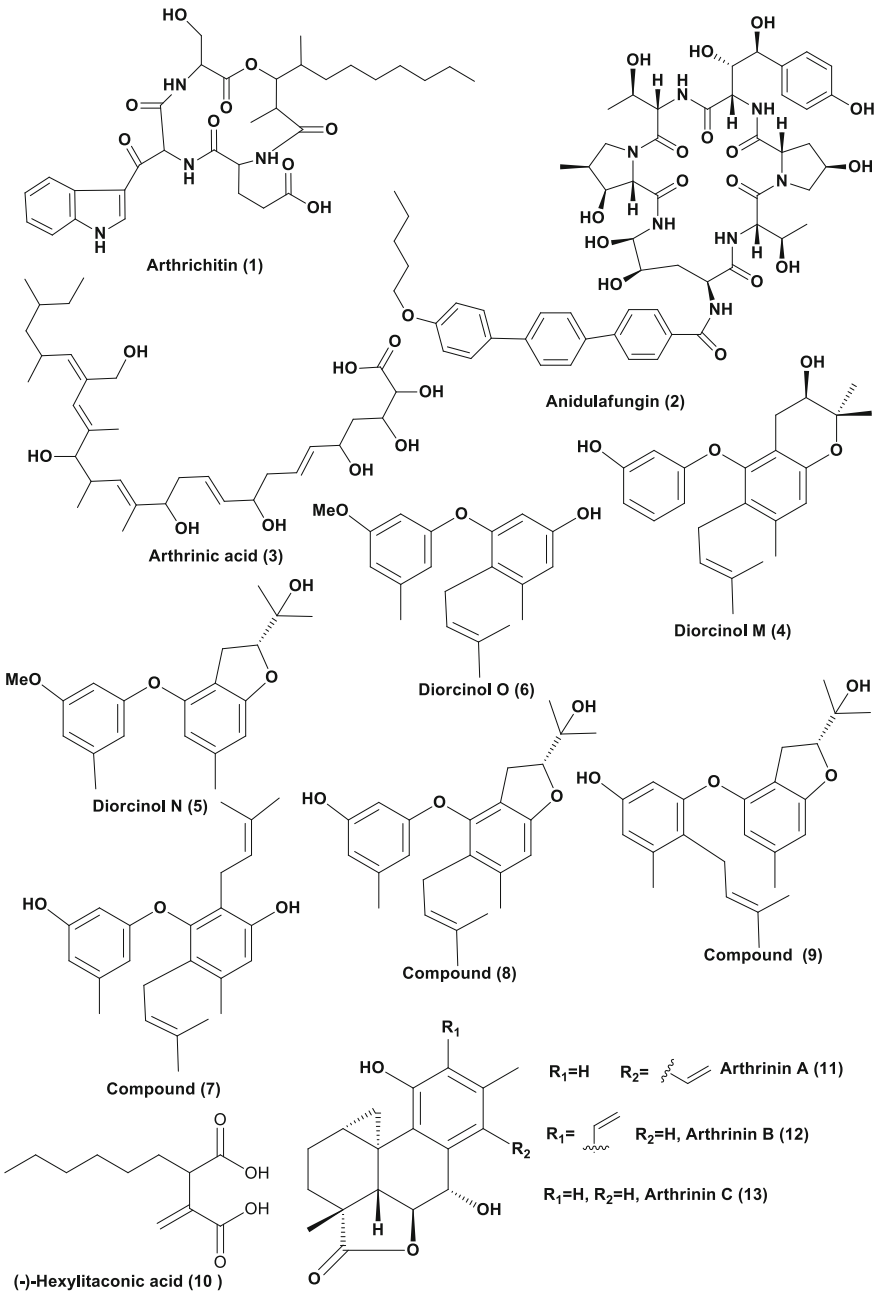

Fig. 9.1 Structural features of metabolites obtained from *Arthrinium* spp. (1–13)

activity to be employed in clinical studies, it needs further derivatization to improve the efficiency [21].

A novel compound, named arundifungin **(2)** (Fig. 9.1), was purified from *Arthrinium arundinis* from Costa Rica. Arundifungin is a new class of inhibitors of glucan synthesis as it prevents normal polarized growth, which leads to short, stunted and highly branched hyphae with bipolar or vesicular tips, swollen germ tubes and balloon-like cells similar to the compound echinocandins. Arundifungin displayed antifungal activity against most of the *Candida* spp. assessed [e.g. *C. albicans* (CLY539), *Candida albicans* (MY1055), *C. glabrata* (MY1381), *C. parapsilosis* (MY1010), *C. pseudotropicalis* (MY2099), *C. tropicalis* (MY1012), *C. tropicalis* (MY1124) and *C. krusei* (CLY549)] with MIC ranging 2–8 μg/ml. Compound **(2)** also showed inhibition of *Aspergillus fumigatus* with 1 μg/ml, while it has very low activity against *Cryptococcus* spp. and an unencapsulated form (MY2062) of strains. However, the arundifungin did not show any antibacterial activity up to 64 μg/ml [20].

Arthrinic acid **(3)** (Fig. 9.1) was isolated from *Arthrinium phaeospermum*. Its structure was elucidated by the NMR spectroscopic studies as (6E,10E,14E,18E,20E)-2,3,5,9,13,17-hexahydroxy-20-(hydroxymethyl)-14,16,18,22,24-pentamethylhexacosa-6,10,14,18,20-pentaenoic acid. It displayed broad-spectrum antifungal activity against different agricultural fungal target species (e.g. *Botrytis cinerea*, *Diplodia pinea* and *Rhizopus stolonifera*) with MIC of 50 μg/ml [22].

Three new prenylated diphenyl ethers diorcinol M–O **(4–6)** together with previously reported known analogues **(7–9)** (Fig. 9.1) were purified from the leaf endophytic fungus *Arthrinium arundinis* (TE-3) of tobacco (*Nicotiana tabacum* L.). Compounds **(4)** and **(5)** exhibited differential antifungal activity against *Mucor hiemalis* (MIC of 8 and 4 μg/mL, respectively). Compounds **(8)** as well as **(9)** displayed inhibition against *Alternaria alternata* (MIC of 8 μg/mL) [23].

9.2.2 Anticancer Activity

From *Arthrinium* sp. isolated from marine sponges (Toyama Bay, Japan), (−)-hexylitaconic acid **(10)** (Fig. 9.1) was extracted. The structure of this compound was elucidated using spectroscopic data, and ELISA technique demonstrated binding with p53-HDM2 (IC_{50}, 50 μg/mL) [11].

From *Arthrinium* sp. obtained from the Mediterranean sponge (*Geodia cydonium*), five new diterpenoids arthrinins A–C **(11–13)** (Fig. 9.1) and D **(14)** and myrocin D **(15)** along with previously reported five known compounds myrocin A **(16)**, norlichexanthone **(17)**, anomalin A **(18)**, decarboxycitrinone **(19)** and 2,5-dimethyl-7-hydroxychromone **(20)** (Fig. 9.2) were obtained. Compound **(17)** showed anticancer activity against four cell lines (L5178Y, K562, A2780 and A2780 CisR) with IC_{50} 1.16, 253.50, 68.2 and 74.0 μM, respectively.

Compound anomalin A **(18)** displayed anticancer potential against cell line L5178Y with IC_{50} 0.40 μM. Compounds **(17** and **18)** exhibited inhibitory activity

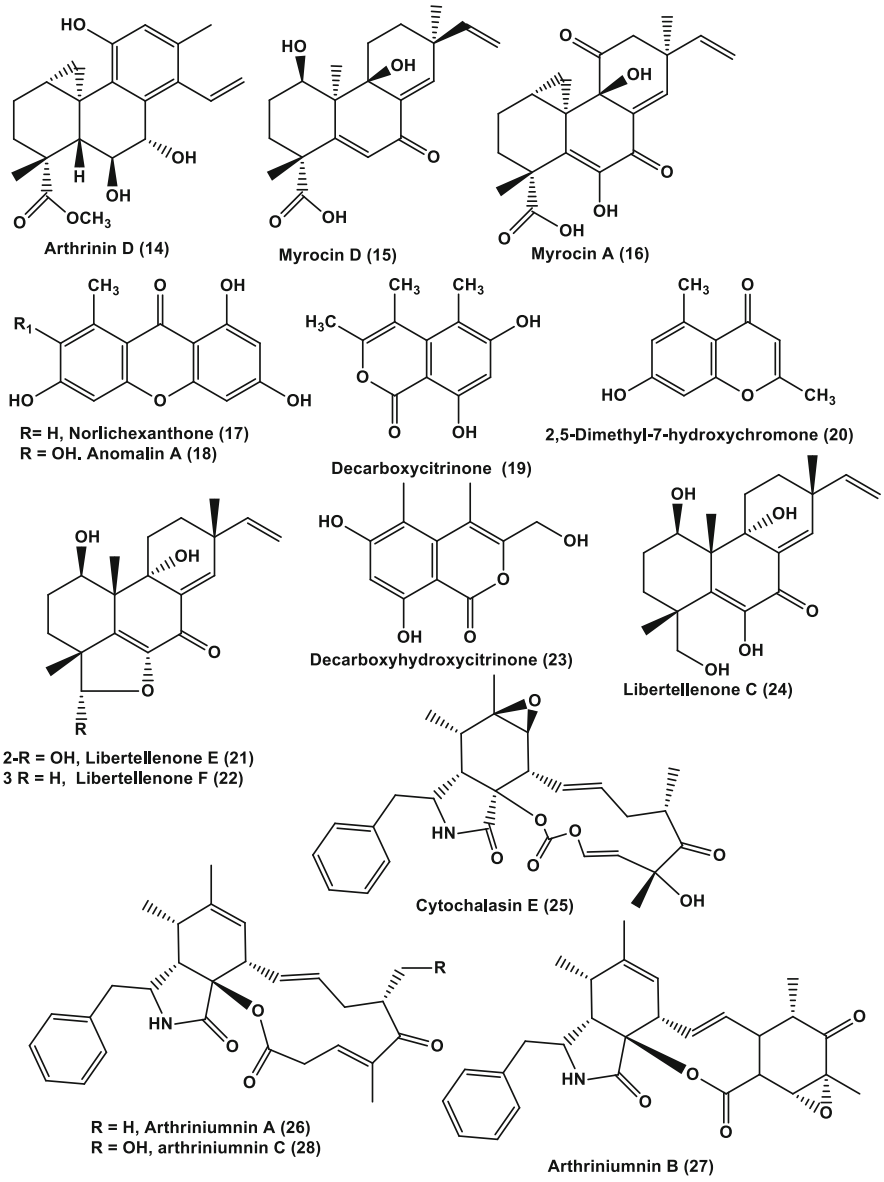

Fig. 9.2 Structural features of metabolites obtained from *Arthrinium* spp. (14–28)

in vitro against three protein kinases (aurora-B, PIM1 and VEGF-R2) with IC_{50} ranging from 0.3 to 11.7 μM, respectively. Compounds **(18, 15** and **16)** showed inhibition of sprouting of VEGF-A-dependent endothelial cells with IC_{50} 1.8, 2.6 and 3.7 μM, respectively, while the compound norlichexanthone **(16)** was inactive [14].

From *Arthrinium sacchari* isolated from an unidentified marine sponge (Atamishi, Shizuoka Prefecture, Japan), three new diterpenes myrocin D **(15)**, libertellenone E **(21)** and libertellenone F **(22)**, another new isocoumarin decarboxyhydroxycitrinone **(23)**, and earlier reported diterpenes myrocin A **(16)** and libertellenone C **(24)** with a known cytochalasin E **(25)** (Fig. 9.2) were purified. Compounds **(16, 23–24)** exhibited inhibitory action against HUVECs and HUAECs with IC_{50} between 7.6 and 68.7 μM. Compound **(25)** also showed inhibition against HUVECs and HUAECs with IC_{50} 0.0114 and 0.0110 μM, respectively, while the positive control Ki8751 showed inhibition against HUVECs as well as HUAECs with IC_{50} 1.0–2.0 μM [24].

From *Arthrinium arundinis* (ZSDS1-F3) associated with a sponge (*Phakellia fusca*), four new cytochalasins, arthriniumnins A–C **(26–28)** (Fig. 9.2) and D (**29**), and a new natural product, ketocytochalasin **(30)**, along with known cytochalasin analogues, viz. cytochalasin K **(31)**, cytochalasin Z16 **(32)**, rosellichalasin **(33)**, 10-phenyl-[12]-cytochalasin Z16 **(34)** as well as cytochalasin Z17 **(35)** (Fig. 9.3) were identified. Compounds **(31** and **34)** displayed activity against cancer cell lines (K562, A549, Huh-7, H1975, MCF-7, U937, BGC823, HL60, Hela and MOLT-4) possessing IC_{50} range between 1.13 and 47.4 μM [25].

Compounds 3,4,5-trimethyl-6-methoxy-8-hydroxyisocoumarin named arthrinisocoumarin A **(36)**, decarboxycitrinone **(19)**, 4-hydroxy-17R-methylincisterol **(37)**, 4-hydroxy-3-methoxybenzoic acid **(38)**, dibutyl phthalate **(39)**, flemingipanic acid **(40)**, indole-3-carboxy acid **(41)** (Fig. 9.3), ergosterol peroxide **(42)**, p-hydroxybenzoic acid **(43)** and 4-hydroxybenzaldehyde **(44)** (Fig. 9.4) were purified from the endophytic fungus *Arthrinium* sp. (A092) isolated from *Uvaria microcarpa*. Compounds **(37)** were found active against SF-268 and MCF-7 cells with IC_{50} 63.8 and 57.2 μM/L, respectively. Compound **(42)** exhibited cytotoxic potential against cell lines SF-268, MCF-7 and NCI-H460 with IC_{50} 50.6, 32.3 and 39.0 μM, respectively [26].

From *A. arundinis* (ZSDS1-F3) adapting an approach of one strain-many compounds, a new phenethyl 5-hydroxy-4-oxohexanoate **(45)** and five known compounds trans-(3R,4S)-(−)-4-hydroxymellein **(46)**, cis-(3R,4R)-(−)-4-hydroxymellein **(47)**, R-(−)-5-hydroxymellein **(48)**, decarboxycitrinone **(19)** and altechromone A **(49)** were purified (Fig. 9.4). Compounds **(46** and **47)** were active against many cancer cell lines (HeLa, DU145, U937 and HL60) with IC_{50} range from 3.8 to 41.1 μM [8].

Arthrinium sp. isolated from sponges yielded 2 new meroterpenoidal alcohols spiroarthrinols A **(50)** and B **(51)** along with 18 recognized compounds: griseofulvin **(52)** and its dechloroderivative **(53)**, 7-hydroxy-2-(2- hydroxypropyl)-5-methylchromone **(54)**, emodin **(55)**, chrysophanol **(56)**, endocrocin **(57)** (Fig. 9.4), 3,8-dihydroxy-6-methoxy-8-methylxanthone **(58)**, norlichexanthone **(59)**, 2 (4-hydroxyphenyl)ethylacetate **(60),** 2-(4-hydroxyethyl) phenol (tyrosol) **(61)**, phomonitroester **(62)**, 3-nitropropionic acid **(63)**, 3,4-dimethoxybenzoic acid **(64)**, 1:1 mixture of 3-phenylpropane-1,2-diol **(65),** 4-hydroxyphenylacetic acid **(66)**, 8-O-4 dehydrodiferulic acid **(67)**, 3β,22-dihydroxylanosta-7,9(11),24-triene **(68)**

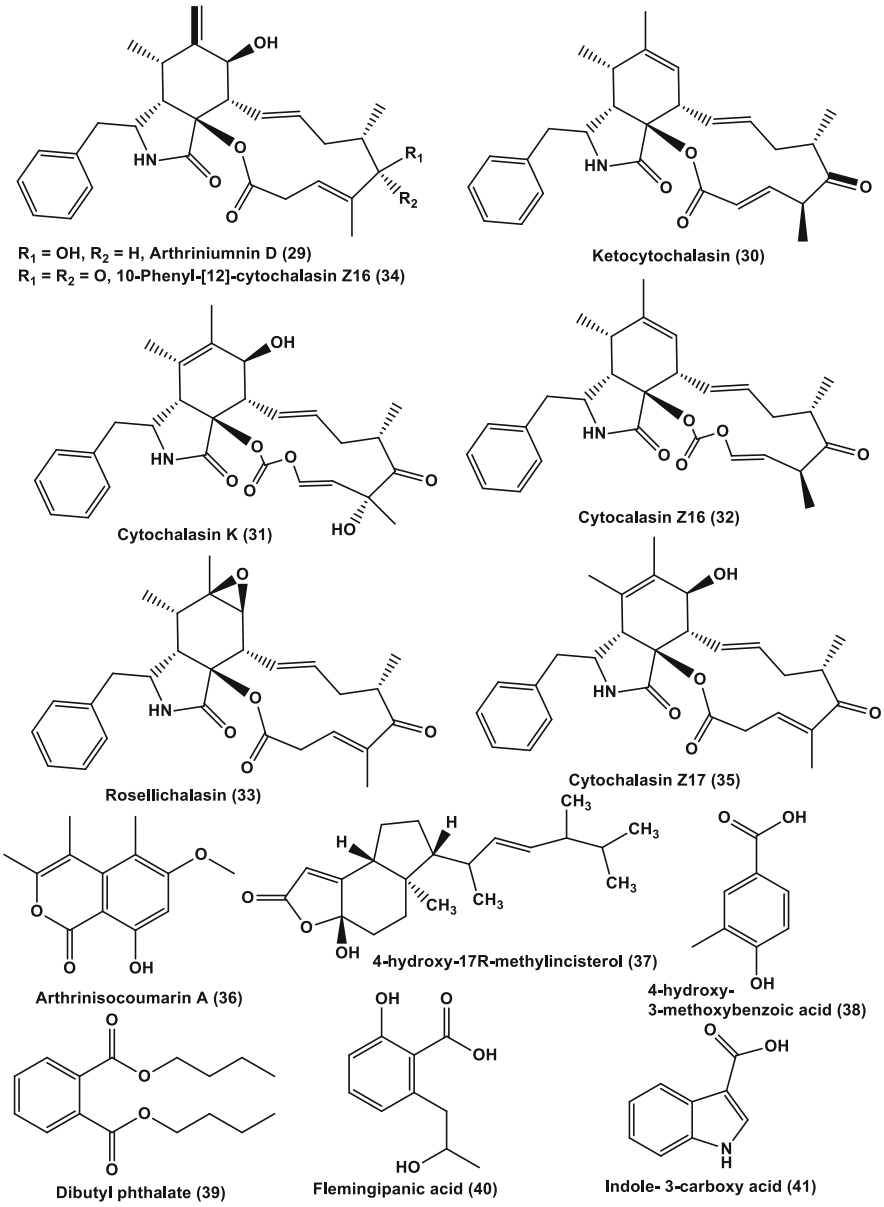

Fig. 9.3 Structural features of metabolites obtained from *Arthrinium* spp. (29–41)

and dankasterone A **(69)** (Fig. 9.5). In an antiproliferative assay, compounds **(50–69)** did not show significant cytotoxicity against Caco-2 cell lines [5].

From the endophytic *Arthrinium arundinis* (TE-3), prenylated diphenyl ether diorcinol N **(5)** together with known analogues **(8–9)** were purified. Compounds

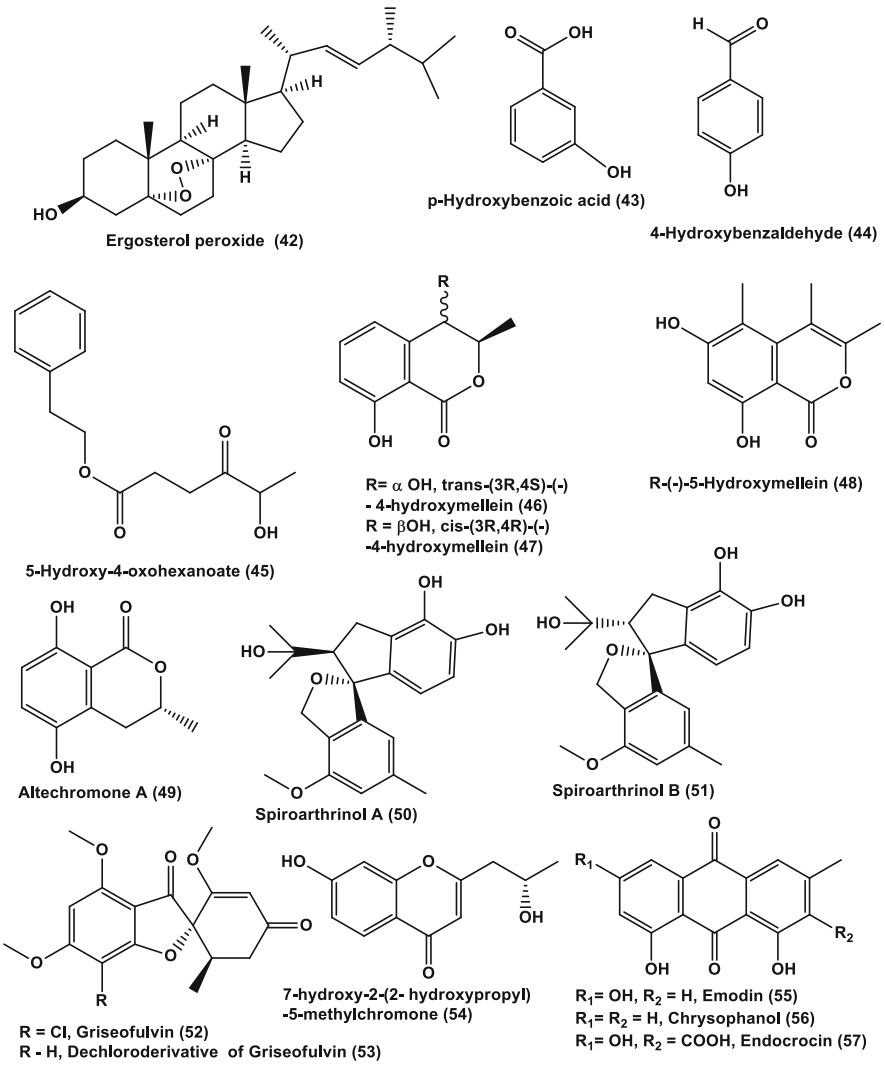

Fig. 9.4 Structural features of metabolites obtained from *Arthrinium* spp. (42–57)

(5, **8** and **9)** showed an average cytotoxicity on the cell line THP-1 with IC_{50} 40.2, 28.3 and 25.9 μM, respectively [23]. Further, diorcinol N **(5)** exhibited anticancer potential against A3 cells with an IC_{50} 16.31 μM. Transcriptome analysis revealed that diorcinol N **(5)** modulated the transcriptome of A3 cells. In total, 9340 genes with differential expression were found, among them 4378 downregulated as well as 4962 upregulated genes responsible in the processes of autophagy, cell cycle and replication of DNA. Furthermore, it was shown that diorcinol N **(5)** induces autophagy, arrests the cell cycle in the G1 or S phase (through the pathways p21,

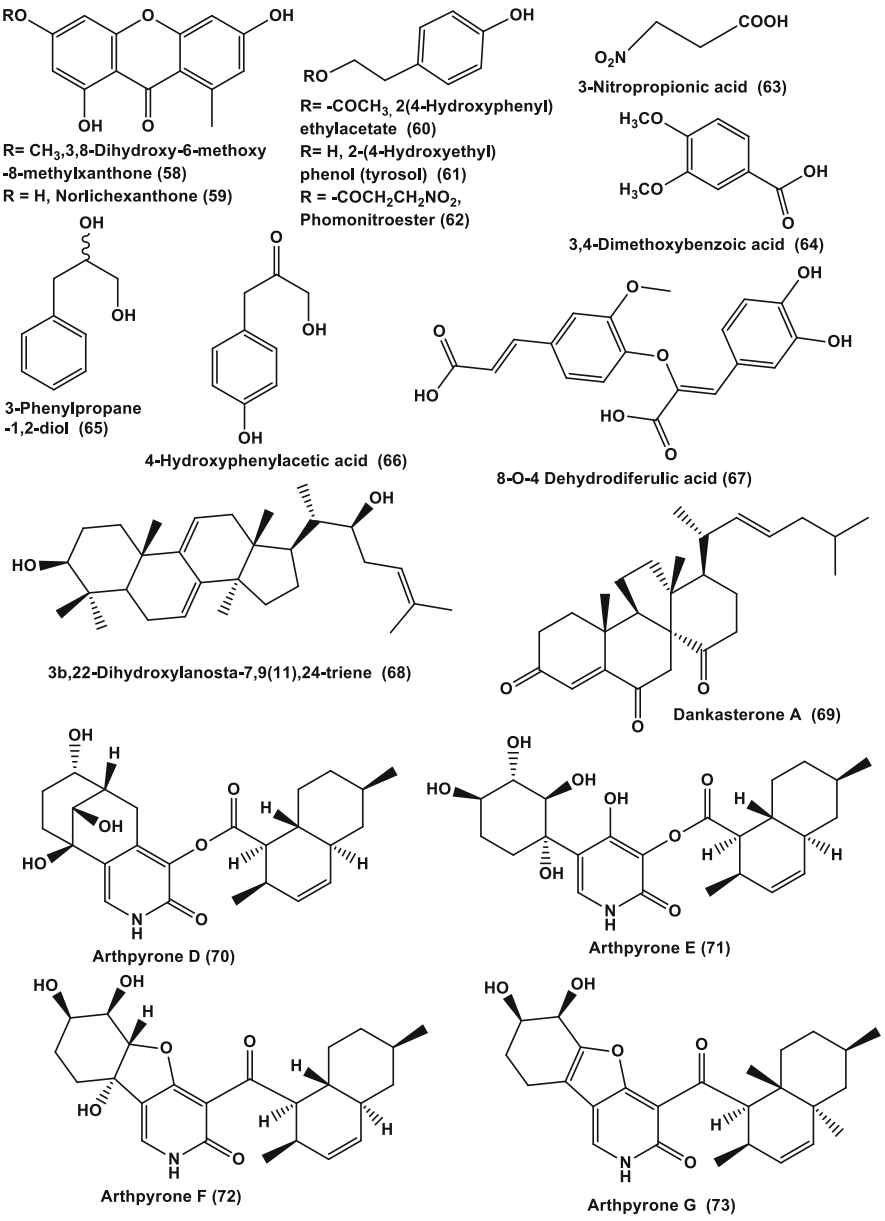

Fig. 9.5 Structural features of metabolites obtained from *Arthrinium* spp. (58–73)

p27, TGF-β and the PI3K/Akt/mTOR) and downregulates autophagy- as well as cell cycle-related genes of A3 cells. Through labelling A3 cells (by acridine orange/ethidium bromide, monodansylcadaverine and Hoechst 33,258) and using transmission electron microscopy, it has been apparent that the DN increased the

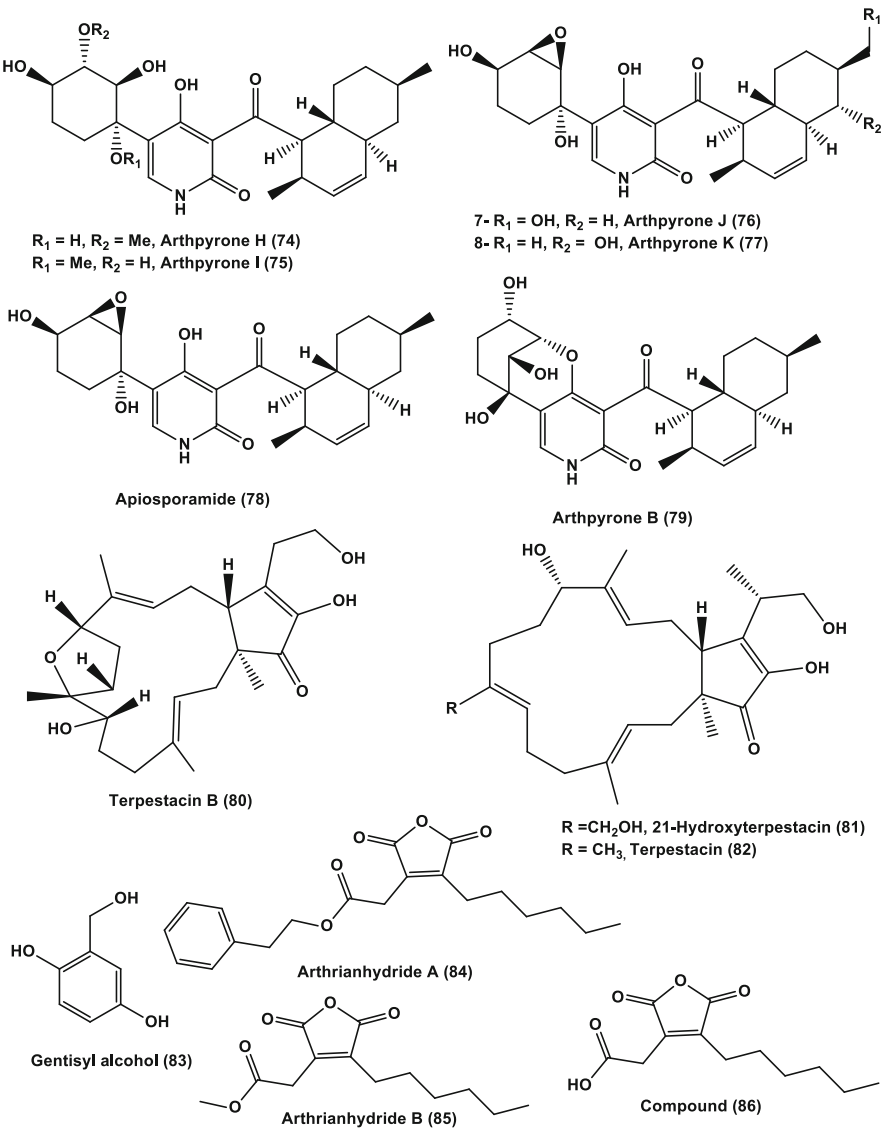

Fig. 9.6 Structural features of metabolites obtained from *Arthrinium* spp. (74–86)

permeability of plasma membrane, vacuolation, structural disorganization and production of autophagosome [15].

Eight novel 4-hydroxy-2-pyridone alkaloids arthpyrones D–G **(70–73)** (Fig. 9.5) and arthpyrones H–K **(70–77)** with two identified analogues of apiosporamide **(78)** and arthpyrone B **(79)** (Fig. 9.6) were yielded from *Arthrinium* sp. (UJNMF0008) isolated from the deep sea. Compounds **(70** and **71)** with an ester functionally linking the pyridone as well as decalin moieties that were first reported among this

class of metabolites, whereas **(72** and **73)** incorporated a rare natural hexa- or tetrahydrobenzofuro[3,2-c]pyridin-3(2H)-one motif. Compound **(78)** was found active against U2OS and MG63 cell lines with IC_{50} 19.3 and 11.7 μM, respectively [33]. It is reported that compound **(78)** prevented the proliferation by cell cycle arrest in the G0 or G1 phase, which leads to apoptosis of OS cells. Furthermore, inhibition of the PI3K/Akt signalling pathway as well as stimulation of the caspase-mediated apoptotic pathway [44] demonstrated its role in enhancing tumour cell apoptosis.

From *Arthrinium* sp. (5XNZ5-4) obtained from the digestive tissue of marine crabs (Zhairuoshan Island, Zhejiang Province, China), two new sesterterpenes, terpestacin B **(80)** and 21-hydroxyterpestacin **(81)**, along with the similar known compound terpestacin **(82)** were derived. Compounds **(80–82)** did not show any antibacterial and cytotoxic activity [27].

Gentisyl alcohol **(83)** (Fig. 9.6) was purified from *Arthrinium* sp. associated with *Sargassum fulvellum.* Compound **(83)** suppresses propagation and induces cell death through DNA fragmentation in ES2 and OV90 cells. It was established in gentisyl alcohol-treated ovarian cancer cells, accumulation of sub-G1 cells as well as loss of mitochondrial membrane potential was by calcium deregulation. Gentisyl alcohol increased signal transduction in the MAPK and PI3K/AKT pathways [28].

From the grasshopper-associated *Arthrinium* sp. (NF2410), arthrianhydride A **(84)** and B **(85)**, two novel 2-carboxymethyl-3-hexyl-maleic anhydride derivatives, along with three known compounds, **(86)** (Fig. 9.6) and **(87, 88)** (Fig. 9.7), were purified. Compounds **(84)** and **(85)** showed average inhibition against SHP2 and an inhibitory rate of 12.80 and 63.02%, respectively, when the concentration of compounds reached 30 μM [29].

A new 2,3-epoxy naphthoquinol (6R,7R,8R)-theissenone A **(89)** along with two recognized compounds (6S,7R,8R)-theissenone **(90)** and arthrinone **(91)** (Fig. 9.7) were purified from the mangrove endophytic fungus *Arthrinium marii* (M-211). Compound **(89)** possesses an oxatricyclo[5.4.0.03,5]undeca-trien-2-one skeleton. Compounds **(89–91)** displayed cytotoxic action on the H4IIE rat hepatoma cells with IC_{50} 67.5, 46.6 and 13.4 μM, respectively [30].

From *Arthrinium* sp. (UJNMF0008A) isolated from the South China Sea, a novel pyridone alkaloid named arthpyrone L **(92)** (Fig. 9.7), a 4-hydroxy-2-pyridone alkaloid, was obtained. Compound **(92)** displayed antiproliferative activity against cell lines MG63, U2OS, A549, MCF-7 and MDA-MB-231 with IC_{50} values of 7.14, 13.34, 17.56, 22.33 and 24.05 μM respectively. The positive control doxorubicin displayed activity on cell lines MG63, U2OS, A549, MCF-7 and MDA-MB-231 with IC_{50} 3.56, 11.17, 2.40, 1.94 and 2.93 nM, respectively. Compound (**92**) induces apoptosis in the cell line MG63, resulting in cell cycle arrest (G0/G1 phase). Besides, cell death was also caused by activation of the caspase-regulated apoptotic pathway as well as inhibition of the signalling pathway PI3K/Akt [31].

Two novel flavonoids 2,3,4,6,8-pentahydroxy-1-methylxanthone (**93**) and arthone C (**94**) (Fig. 9.7) were isolated from the deep-sea *Arthrinium* sp. and effectively scavenged ROS in vitro. Compounds **(93** and **94)** inhibited ox-LDL-induced cell death and expression of adhesion factors in umbilical vein vascular

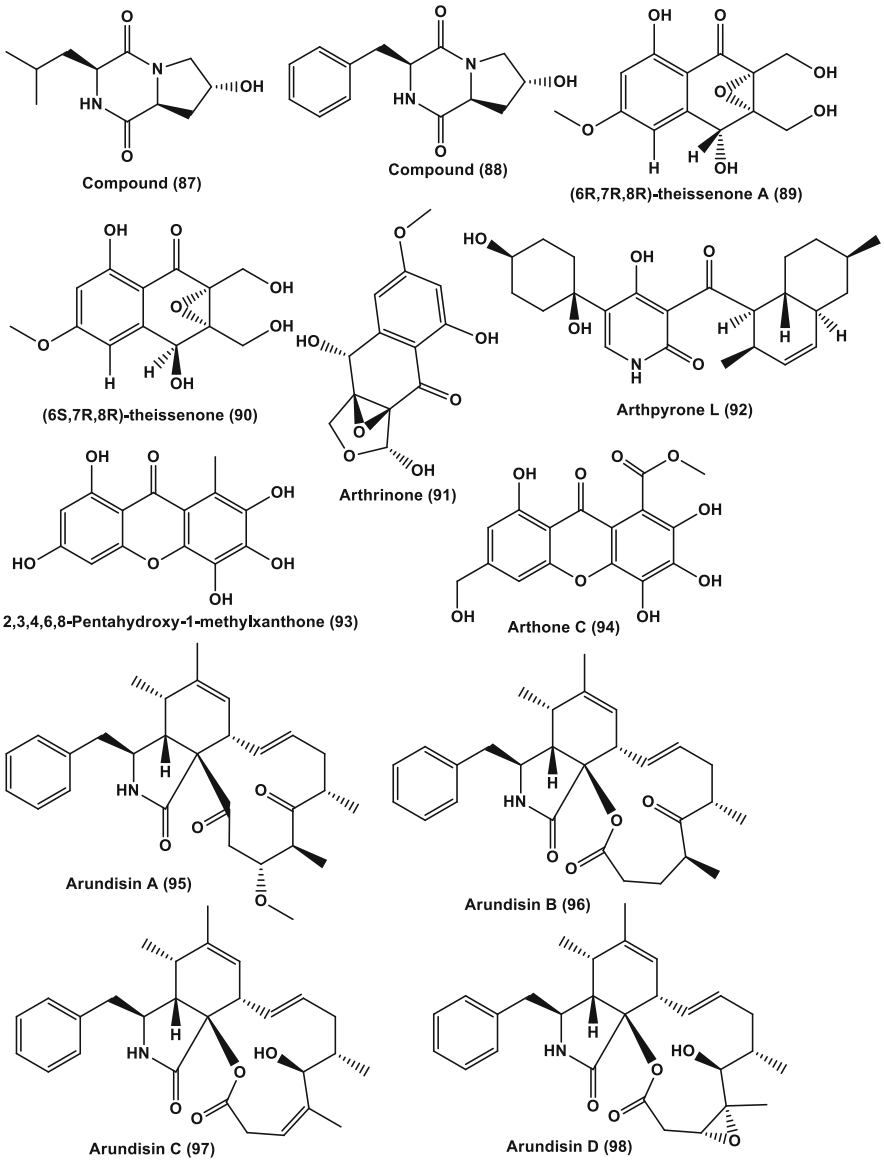

Fig. 9.7 Structural features of metabolites obtained from *Arthrinium* spp. (87–98)

endothelial cells of humans (HUVECs). Mechanistic studies revealed that such compounds considerably inhibit the increase of ROS levels and induce nuclear translocation of NF-κB by ox-LDL. Compounds **(93** and **94)** were also known to activate the transfer of Nrf2 into nuclei and improve the expression of its downstream antioxidant gene (HO-1) by induction of phosphorylation of AKT in HUVECs. Interestingly, the AKT inhibitor (MK-2206 2HCl) or knockdown of

Nrf2 by RNA interference showed inhibitory to these compounds on ox-LDL-induced cell death in HUVECs. Knockdown of Nrf2 terminated the impacts of the compounds on ox-LDL-induced increase of ROS quantity as well as the translocation of NF-κB into nuclei. It was reported that compounds **(93** and **94)** were also capable of protecting endothelial cells against ox-LDL-induced oxidative stress by triggering the pathway of AKT/Nrf2/HO-1 [32].

Six undescribed cytochalasans, arundisins A–D **(95–98)** (Fig. 9.8) and arundisins E–F **(99–100)**, and twelve known compounds **(101–106)** and **(107–111)** (Fig. 9.9) and ergosterol peroxide **(42)** were purified from *Arthrinium arundinis* DJ-13. Compounds **(95** and **96)** displayed cytotoxic action on the cell line MCF-7 with IC_{50} 18.82 and 15.20 μM, respectively [19].

9.2.3 Antibacterial Activity

New 4-hydroxy-2-pyridone alkaloids arthpyrones F–G (**72–73**) (Fig. 9.5) and arthpyrones H–I (**74–75**) and known analogues apiosporamide (**78**) (Fig. 9.6). were identified in *Arthrinium* sp. (UJNMF0008) isolated from the deep sea. Compounds **(72–75, 78)** displayed average to remarkable action on *Mycobacterium smegmatis* and *Staphylococcus aureus* with IC_{50} range of 1.66 to 42.8 μM [33].

An undescribed cytochalasin and arundisins D and F **(98, 100)** (Fig. 9.8) were identified, from *Arthrinium arundinis* (DJ-13). Compound **(100)** showed significant antibacterial activity on *Escherichia coli* with MIC of 8.00 μg/mL (standard kanamycin showed MIC of 2.00 μg/mL), while arundisin D (**98**) showed mean antifungal activity on *Candida albicans* with MIC of 32.00 μg/mL (standard nystatin had MIC of 1.00 μg/mL) [19].

From the *Arthrinium* sp. isolated from the South China Sea, a novel griseofulvin derivative, (+)-5-chlorogriseofulvin **(112)**, along with two known analogues griseofulvin **(113)** and dechlorogriseofulvin **(114)** (Fig. 9.9) was identified. Compounds **(112–114)** showed poor action on *Micrococcus luteus* with MIC of 100 μM. Compound **(112)** also displayed an average lethal effect on the brine shrimp (*Artemia salina*) with LC_{50} 25 μg/mL [12].

9.2.4 Anti-inflammatory Activity

From the *Arthrinium* sp. (ZSDS1-F3) isolated from sponges, a novel naphthalene derivative, 1,8-dihydroxynaphthol-1-O-α-l-rhamnopyranoside **(115)**, along with a recognized α-dibenzopyrone, alternariol **(116)**, and five known xanthones, 1,3,6-trihydroxy-8-methylxanthone **(117)**, 1,3,5,6-tetrahydroxy-8-methylxanthone **(118)**, anomalins A **(119)** and B **(120)** and fischexanthone **(121)** (Fig. 9.9), were recognised. Compound **(117)** displayed average COX-2 inhibition, with IC_{50} 12.2 μM [16].

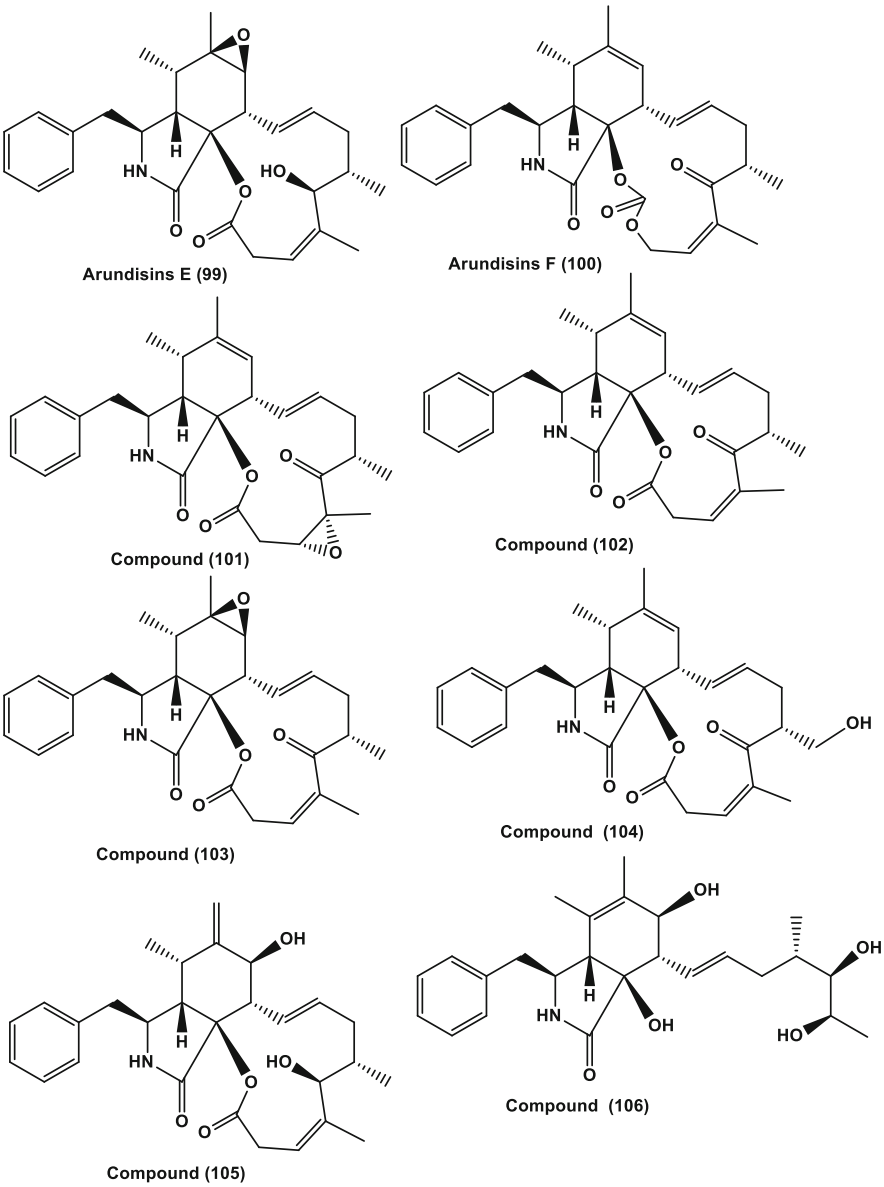

Fig. 9.8 Structural features of metabolites obtained from *Arthrinium* spp. (99–106)

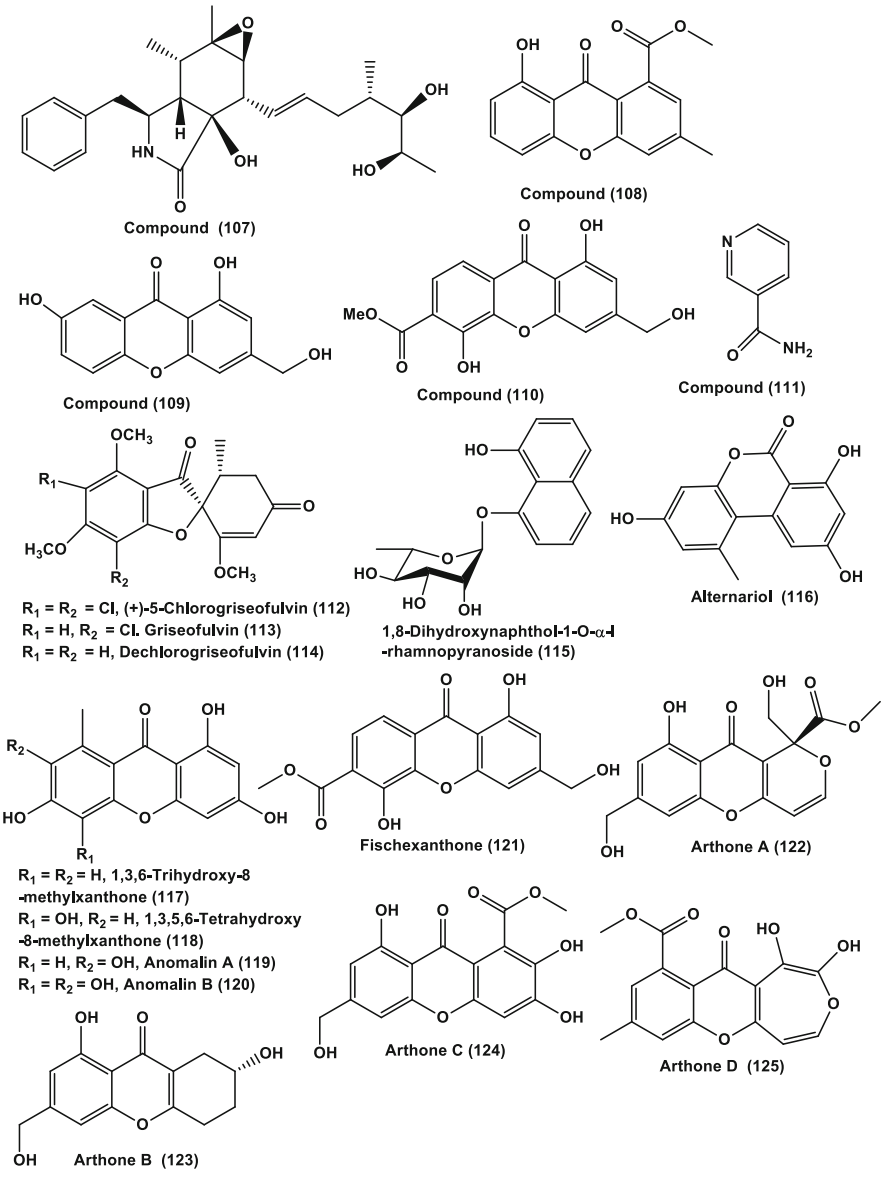

Fig. 9.9 Structural features of metabolites obtained from *Arthrinium* spp. (107–125)

9.2.5 Antioxidant Activity

From *Arthrinium* sp. (UJNMF0008) isolated from the deep sea, five novel chromone derivatives, arthones A–E **(122–125)** (Fig. 9.9) and arthone E (**126**), along with eight known biogenetically connected cometabolites AGI-B4 **(127)**, 2,3,4,6,8-pentahydroxy-1-methylxanthone **(128)**, sydowinin A **(129)**, sydowinin B **(130)**, conioxanthone A **(131)**, engyodontiumone B **(132)** and 8-hydroxy-3-hydroxymethyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ester **(133)** (Fig. 9.10) and 1,3,6-trihydroxy-8-methylxanthone (7) **(120)**, were identified. Compounds **(124** and **128)** displayed potent antioxidant activity with IC_{50} of 16.9 to 18.7 μM in assays of DPPH and ABTS radical scavenging [17].

Arthrinium sp. associated with the seaweed *Sargassum fulvellum* was the source of gentisyl alcohol **(83),** which displayed antioxidant activity with IC_{50} 26.43 and 28.74 μM, respectively, in assays ABTS and DPPH radical scavenging (positive control, ascorbic acid showed IC_{50} 77.79 and 38.6 μM, respectively) [18].

9.2.6 Wormicidal Activity

From endophytic *Arthrinium* sp., R-(−)-mellein **(134)** and cis-(3R,4R)-4-hydroxymellein **(135)** were obtained. Compounds **(134** and **135)** caused the mortality of 100% of *Schistosoma mansoni* adult worms between 200 and 50 μg mL, respectively. An ultrastructure assessment showed that the tegument can be one of the targets of compounds **(134)** [34].

9.2.7 Some Other Compounds from *Arthrinium* spp.

From *A. arundinis* (ZSDS1-F3), three new 4-hydroxy-2-pyridone alkaloids, arthpyrones A–C **(136–138)**, along with the recognized pyridone alkaloid analogue N-hydroxyapiosporamide **(139)** (Fig. 9.10), were identified. Compounds **(136** and **137)** with a 2-pyridone core with a decalin moiety connected through a carboxide bridge have a unique oxabicyclo[3.3.1]nonane ring system, which was infrequently discovered [35].

From endophytic *Arthrinium phaeospermum* (JS 0567), 2, 3, 6, 8-tetrahydroxy-1-methylxanthone (**140**), 2, 3, 4, 6, 8-pentahydroxy-1-methylxanthone (**141**), 3, 4, 6, 8-tetrahydroxy-1-methylxanthone (**142**), 3, 6, 8-trihydroxy-1-methylxanthone (**143**), 2, 4,2′,4′,6′-pentahydroxy-6-methylbenzophenone (**144**) and 5,7-di hydroxy-3-methylphthalide (**145**) (Fig. 9.10) were purified [48].

Four polyketides were purified from endophytic *Arthrinium* sp. (JS420) isolated from a halophyte (*Suaeda japonica*): tyrosol **(61)**, (3R,4R)-(−)-4-hydroxymellein **(146)**, (3R,4S)-(−)-4-hydroxymellein **(147)** and 1-phenyl-1,2-ethanediol **(148)** (Fig. 9.10) [36].

Arthrinium arundinis endophytic fungus associated with the leaves of *Anoectochilus roxburghii* was the source of a new xanthone, 2,8-dihydroxy-9-oxo-9H-xanthene-6-carboxylic acid **(149),** and six compounds, 2,8-dihydroxy-1-

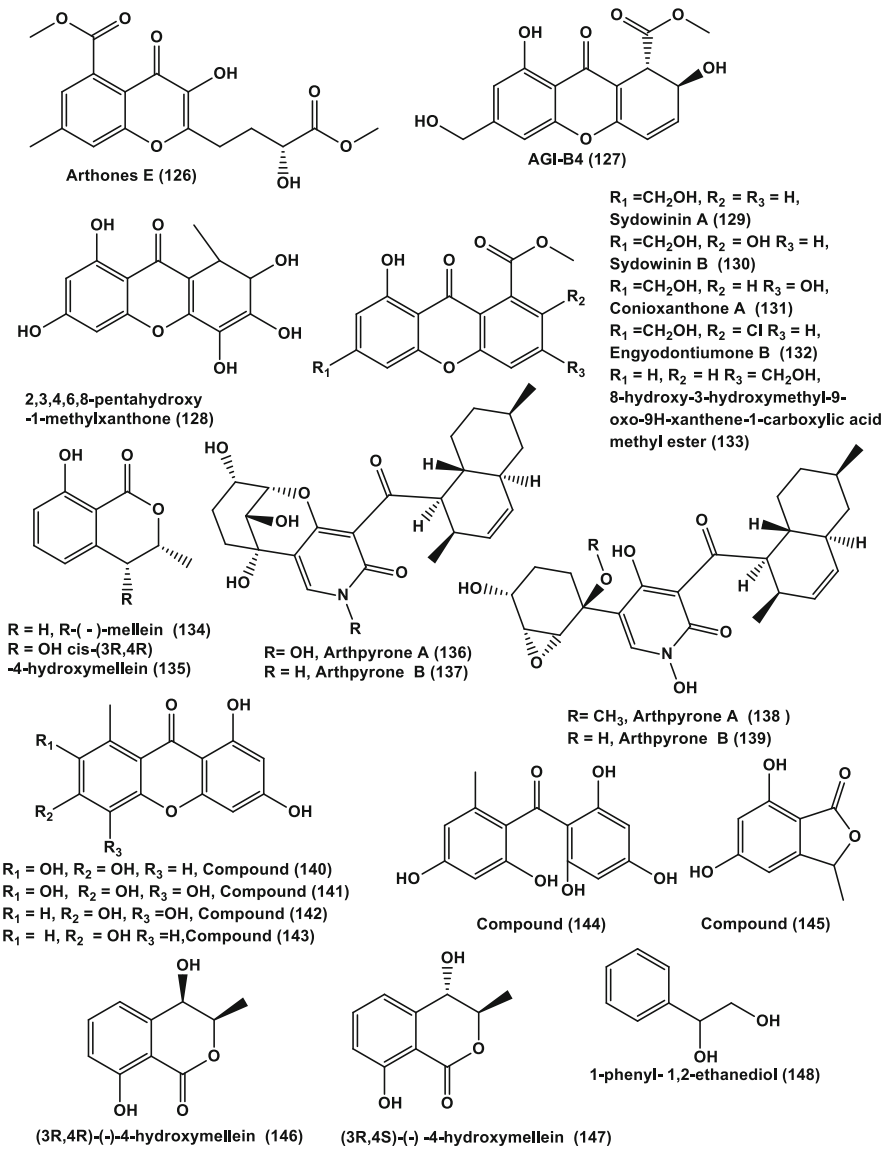

Fig. 9.10 Structural features of metabolites obtained from *Arthrinium* spp. (126–148)

methoxycarbonyl-9-oxo-9H-xanthene-6-carboxylic acid **(150)**, 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether **(151)**, 1,6-dihydroxy-3-methoxy-8-methylxanthone **(152)**, formoic acid A **(153)** (Fig. 9.11), decarboxycitrinone **(19)** and 1,3,6-trihydroxy-8-methylxanthone **(120)** [37].

Morishita et al. [38] evaluated the use of plant hormone to activate silent polyketide biosynthesis in *Arthrinium sacchari*, a fungus isolated from spider.

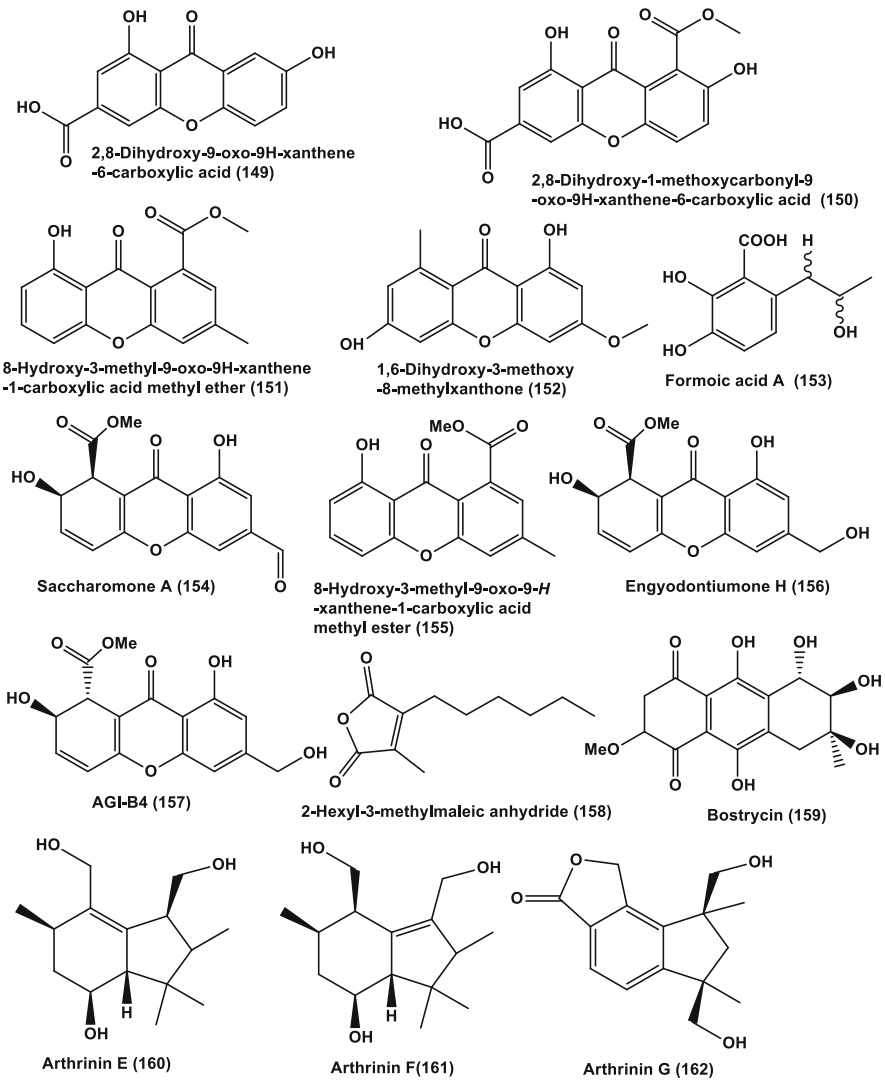

Fig. 9.11 Structural features of metabolites obtained from *Arthrinium* spp. (149–162)

Cytokinins 6-benzylaminopurine (BAP) and forchlorfenuron (FCF) stimulated the production of xanthone derivatives saccharomone A **(154)**, a new engyodontiumone analogue 8-hydroxy-3-methyl-9-oxo9H-xanthene-1-carboxylic acid methyl ester **(155)**, engyodontiumone H **(156)** and AGI-B4 **(157)**. Production of 2-hexyl-3-methylmaleic anhydride **(158)** (Fig. 9.11) was increased by BAP as well as FCF. The FCF significantly stimulated the production of bostrycin **(159)**, an anthraquinone derivative [38]. Both aromatic polyketides might have been biosynthesized through emodin. In the *Arthrinium sacchari* draft genome, clusters having TE-less

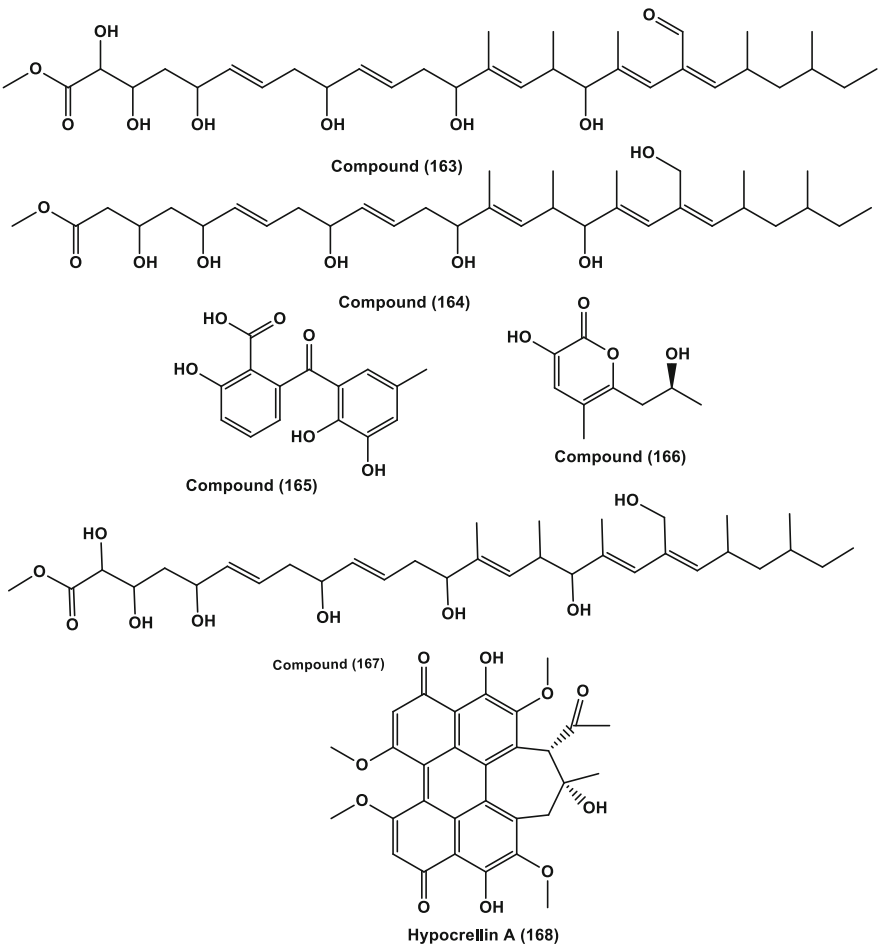

Fig. 9.12 Structural features of metabolites derived from *Arthrinium* spp. (163–168)

NR-PKS and β-lactamase TE were recognized as emodin **(55)** biosynthetic genes with two candidate biosynthetic clusters of genes (anp161 and anp186). Transcriptional studies showed that BAP stimulated the expression of anp161 gene cluster containing the Baeyer-Villiger oxidase, which is one of the key enzymes for the ring reconstruction of anthraquinone to a xanthone. Thus, the BAP-induced production of xanthone derivatives **(154–157)**. The FCF stimulated the silent gene cluster anp186 as well as activated the biosynthesis of bostrycin **(159)** [38]. From *Arthrinium phaeospermum*, bostrycin (**159**) was reported by van Eijk [39].

From endophytic *Arthrinium* sp. (HS66) occurring in the stems of *Isodon xerophilus*, three arthrinins E–G **(160–162)** (Fig. 9.11) and three new sesquiterpenoids having a non-isoprenoid botryane skeleton were purified. Their

structures were determined by extensive spectroscopic techniques. In addition, structure of **(160)** was precisely established by X-ray diffraction, whereas **(161** and **162)** were assessed through quantum chemical calculation of NMR data as well as ECD spectra [40].

Four new polyketides with two arthrinic acid derivatives **(163, 164)**, one phenolic derivative **(165)** and (S)-3-hydroxy-6-(2-hydroxypropyl)-5-methyl-2H-pyran-2-one **(166)** along with one methyl ester of arthrinic acid **(167)** (Fig. 9.12) were obtained from endophytic *Arthrinium* sp. associated with clam worm [41].

From the stroma of *Shiraia bambusicola* as well as *Hypocrella bambusae*, hypocrellin was purified, which is a natural 3,10-xylene-4,9-anthracene derivative with excellent photobiological activities. From endophytic *Arthrinium* sp. (AF-5) from tissues of bamboo, hypocrellin was obtained by incubation with *S. bambusicola* (GDMCC 60438). The yield of hypocrellin A (HA) **(168)** (Fig. 9.12) attained up to 66.75 mg/g carbon source on 84 hr. co-cultivation of the dual strains. It was a fourfold increase by the fermentation with *S. bambusicola*. The microscopic observations showed that the mycelia of the two strains were intertwined with each other to form the mycelium pellets during the co-cultivation. Moreover, the mycelium pellets of the co-culture showed a contracted and slightly damaged morphology. On the addition of H_2O_2 to the fermentation media, the production of HA **(168)** has been increased, which was up to 18.31% [42].

9.3 Biotransformation

From an endophytic *Arthrinium* sp. (GE 1718) in *Panax ginseng*, a β-glycosidase was produced by biotransformation of ginsenoside Rb1 to ginsenoside C-K. A β-glucosidase-producing endophytic *Arthrinium* sp. (GE 1718) hydrolysed the major ginsenosides Rb1 to minor ginsenoside C-K with ginsenoside Rb1 → ginsenoside and Rd → ginsenoside F2 → ginsenoside C-K metabolic pathways [43].

9.4 Conclusions

The present review reveals the fact that different *Arthrinium* spp. possess immense chemical diversity as well as they are prolific producers of several value-added bioactive compounds. These metabolites belong to various classes like peptides, indoles, terpenes, terpenoids, coumarin, xanthone, pyrone and naphthol. The metabolites obtained from this fungus display an extensive range of biological properties (antibacterial, antifungal, anticancer, anti-inflammatory, antioxidant and vermicidal activities). Some compounds, such as arthrichitin and arundifungin, can be potential candidates for further development that includes in vitro and in vivo studies against a large number of pathogens, chemical modification and mode of action. Other compounds like diorcinol N **(5)**, apiosporamide **(78)**, arthpyrone L **(92)** and 2,3,4,6,8-pentahydroxy-1-methylxanthone **(93)** as well as arthone C **(94)**

displayed anticancer activity that needs to be investigated further. We have reported 170 compounds, with a large number of these compounds having not been assessed for any of their biological activities. Since this fungus has been reported from various habitats, extensive work is needed to isolate this fungus from extreme environments. Whole-genome sequencing is necessary for the assessment of their production of novel compounds. There are some reports on the whole-genome sequence of *Arthrinium* sp. [45, 46]. Molecular approaches for yield impovement of omphalotin was done by the transfer of biosynthetic gene clusters to a appropriate vactor. Another approach for producing innovative compounds from *Arthrinium* is the use of precursors of biosynthetic pathways in the culture medium, which may lead to the production of several metabolites through the speedup of biosynthetic pathways [47]. Bioactive metabolites from *Arthrinium* opened up ample opportunities to explore novel compounds as well as different chemical skeletons that may serve as structural prototypes in order to enforce directed therapeutic compounds.

References

1. Schmidt J, Kunze G (1817) Mykologische Hefte. 1. Vossische Buchhandlung, Leipzig
2. Crous PW, Groenewald JZ (2013) A phylogenetic re-evaluation of *Arthrinium*. IMA Fungus 4(1):133–154
3. Kim JJ, Lee SS, Ra JB, Lee H, Huh N, Kim GH (2011) Fungi associated with bamboo and their decay capabilities. Holzforschung 65(2):271–275
4. Wang M, Tan X-M, Liu F, Cai L (2018) Eight new *Arthrinium* species from China. MycoKeys 34(34):1–24
5. Elissawy AM, Ebada SS, Ashour ML, Özkaya FC, Ebrahim W, Singab AB, Proksch P (2017) Spiroarthrinols a and B, two novel meroterpenoids isolated from the sponge-derived fungus *Arthrinium* sp. Phytochem Lett 20:246–251
6. Flewelling AJ, Currie J, Gray CA, Johnson JA (2015) Endophytes from marine macroalgae: promising sources of novel natural products. Curr Sci 109(88):88–111
7. Hong JH, Jang S, Heo YM, Min M, Lee H, Lee Y, Lee H, Kim JJ (2015) Investigation of marine-derived fungal diversity and their exploitable biological activities. Mar Drugs 13(7): 4137–4155
8. Li Y, Wang J, He W, Lin X, Zhou X, Liu Y (2017) One strain-many compounds method for production of polyketide metabolites using the sponge-derived fungus *Arthrinium arundinis* ZSDS1-F3. Chem Nat Compd 53(2):373–374
9. Miao L, Kwong TF, Qian PY (2006) Effect of culture conditions on mycelial growth, antibacterial activity, and metabolite profiles of the marine-derived fungus *Arthrinium* c.f. *saccharicola*. Appl Microbiol Biotechnol 72(5):1063–1073
10. Suryanarayanan T (2012) Fungal endosymbionts of seaweeds. Biology of Marine Fungi Springer 53:53–69
11. Tsukamoto S, Yoshida T, Hosono H, Ohta T, Yokosawa H (2006) Hexylitaconic acid: a new inhibitor of p53–HDM2 interaction isolated from a marine-derived fungus, *Arthrinium* sp. Bioorg Med Chem Lett 16(1):69–71
12. Wei MY, Xu RF, Du SY, Wang CY, Xu TY et al (2016) A new griseofulvin derivative from the marine-derived *Arthrinium* sp. fungus and its biological activity. Chem Nat Compd 52(6): 1011–1014
13. Park MS, Oh S-Y, Lee S, Eimes JA, Lim YW (2018) Fungal diversity and enzyme activity associated with sailfin sandfish egg masses in Korea. Fungal Ecol 34:1–9

14. Ebada SS, Schulz B, Wray V, Totzke F, Kubbutat MH, Müller WE, Hamacher A, Kassack MU, Lin W, Proksch P (2011) Arthrinins A–D: Novel diterpenoids and further constituents from the sponge derived fungus *Arthrinium* sp. Bioorg Med Chem 19(15):4644–4651
15. Yuan XL, Li XQ, Xu K, Hou XD, Zhang ZF, Xue L, Liu XM, Zhang P (2020) Transcriptome profiling and cytological assessments for identifying regulatory pathways associated with diorcinol N-induced autophagy in A3 cells. Front Pharmacol 11:570450
16. Wang JF, Xu FQ, Wang Z, Lu X, Wan JT, Yang B, Zhou XF, Zhang TY, Tu ZC, Liu Y (2014) A new naphthalene glycoside from the sponge-derived fungus *Arthrinium* sp. ZSDS1-F3. Nat Prod Res 28(14):1070–1074
17. Bao J, He F, Yu JH, Zhai H, Cheng ZQ, Jiang CS, Zhang Y, Zhang Y, Zhang X, Chen G, Zhang H (2018b) New chromones from a marine-derived fungus, *Arthrinium* sp., and their biological activity. Molecules *23*(8):1982
18. Heo YM, Kim K, Ryu SM, Kwon SL, Park MY, Kang JE, Hong JH, Lim YW, Kim C, Kim BS, Lee D (2018) Diversity and ecology of marine algicolous *Arthrinium* species as a source of bioactive natural products. Mar Drugs 16(12):508
19. Shu Y, Wang JP, Li BX, Gan JL, Ding H, Liu R, Cai L, Ding ZT (2022) Bioactive cytochalasans from the fungus *Arthrinium arundinis* DJ-13. Phytochemistry 194:113009
20. Cabello AM, Platas G, Collado J, Díez TM, Martín I, Vicente F, Meinz M, Onishi JC, Douglas C, Thompson J, Kurtz MB (2001) Arundifungin, a novel antifungal compound produced by fungi: biological activity and taxonomy of the producing organisms. Int Microbiol 4(2):93–102
21. Vijayakumar EKS, Roy K, Chatterjee S, Deshmukh SK, Ganguli BN, Kogler H, Fehlhaber HW (1996) Arthrichitin a new cell wall active metabolite from *Arthrinium phaeospermum*. J Org Chem 61:6591–6593
22. Bloor S (2008) Arthrinic acid, a novel antifungal polyhydroxyacid from *Arthrinium phaeospermum*. J Antibiot 61(8):515–517
23. Zhang P, Li X, Yuan XL, Du YM, Wang BG, Zhang ZF (2018) Antifungal prenylated diphenyl ethers from *Arthrinium arundinis*, an endophytic fungus isolated from the leaves of tobacco (*Nicotiana tabacum* L.). Molecules 23(12):3179
24. Tsukada M, Fukai M, Miki K, Shiraishi T, Suzuki T, Nishio K, Sugita T, Ishino M, Kinoshita K, Takahashi K, Shiro M (2011) Chemical constituents of a marine fungus, *Arthrinium sacchari*. J Nat Prod 74(7):1645–1649
25. Wang J, Wang Z, Ju Z, Wan J, Liao S, Lin X, Zhang T, Zhou X, Chen H, Tu Z, Liu Y (2015) Cytotoxic cytochalasins from marine-derived fungus *Arthrinium arundinis*. Planta Med 81(02): 160–166
26. Li HH (2016) A new isocoumarin isolated from endophytic fungus *Arthrinium* sp. A092 derived from medicinal plant *Uvaria microcarpa*. Zhong Cao Yao 2016(3):369–373
27. Ye B, Ding W, Wang PM, Xu J (2019) Two new sesterterpenes from marine-derived fungus *Arthrinium* sp. Chem Nat Compd 55(2):281–284
28. Ham J, Lim W, Kim K, Heo YM, Ryu SM, Lee D, Kim JJ, Song G (2019) Gentisyl alcohol inhibits proliferation and induces apoptosis via mitochondrial dysfunction and regulation of MAPK and PI3K/AKT pathways in epithelial ovarian cancer cells. Mar Drugs 17(6):331. https://doi.org/10.3390/md17060331
29. Wei LI, Jing WEI, Dao-Ying CHEN, Mei-Jing WANG, Yang SUN, Fang-Wen JIAO, Rui-Hua JIAO, Ren-Xiang TAN, Hui-Ming GE (2020) Study on the secondary metabolites of grasshopper-derived fungi *Arthrinium* sp. NF2410. Chin J Nat Med 18(12):957–960
30. Sofian FF, Suzuki T, Supratman U, Harneti D, Maharani R, Salam S, Abdullah FF, Yoshida J, Ito Y, Koseki T, Shiono Y (2021) The 2, 3-epoxy naphthoquinol produced by endophyte *Arthrinium marii* M-211. Nat Prod Res:1–7
31. Zhang Y, Li M, Zhang Q, Wang Z, Li X, Bao J, Zhang H (2021) Arthpyrone L, a new pyridone alkaloid from a deep-sea *Arthrinium* sp., inhibits proliferation of MG63 osteosarcoma cells by inducing G0/G1 arrest and apoptosis. Chem Biodivers 18(2):2000639

32. Hou JR, Wang YH, Zhong YN, Che TT, Hu Y, Bao J, Meng N (2021) Protective effect of flavonoids from a deep-sea-derived *Arthrinium* sp. against ox-LDL-induced oxidative injury through activating the AKT/Nrf2/HO-1 pathway in vascular endothelial cells. Mar Drugs 19(12):712
33. Bao J, Zhai H, Zhu K, Yu JH, Zhang Y, Wang Y, Jiang CS, Zhang X, Zhang Y, Zhang H (2018a) Bioactive pyridone alkaloids from a deep-sea-derived fungus *Arthrinium* sp. UJNMF0008. Mar Drugs 16(5):174
34. Ramos HP, Simão MR, de Souza JM, Magalhães LG, Rodrigues V, Ambrósio SR, Said S (2013) Evaluation of dihydroisocoumarins produced by the endophytic fungus *Arthrinium* state of *Apiospora montagnei* against *Schistosoma mansoni*. Nat Prod Res 27(23):2240–2243
35. Wang J, Wei X, Qin X, Lin X, Zhou X, Liao S, Yang B, Liu J, Tu Z, Liu Y (2015) Arthpyrones A–C, pyridone alkaloids from a sponge-derived fungus *Arthrinium arundinis* ZSDS1-F3. Org Lett 17(3):656–659
36. Choi HG, Shim SH (2017) Secondary metabolites produced by an endophytic fungus *Arthrinium* sp. JS420 of halophytic plant *Suaeda japonica*. Korean. J Pharmacogn 48(1):1–4
37. Liao ZJ, Tian WJ, Liu XX, Jiang X, Wu Y, Lin T, Chen HF (2018) A new xanthone from an endophytic fungus of *Anoectochilus roxburghii*. Chem Nat Compd 54(2):267–269
38. Morishita Y, Okazaki Y, Luo YY, Nunoki J, Taniguchi T, Oshima Y, Asai T (2019) Use of plant hormones to activate silent polyketide biosynthetic pathways in *Arthrinium sacchari*, a fungus isolated from a spider. Org Biomol Chem 17(4):780–784
39. van Eijk GW (1975) Bostrycin, a tetrahydroanthraquinone pigment and some other metabolites from the fungus *Arthrinium phaeospermum*. Experientia 31(7):783–784
40. Su XZ, Tang JW, Hu K, Li XN, Sun HD, Puno PT (2020) Arthrinins E–G, three botryane sesquiterpenoids from the plant endophytic fungus *Arthrinium* sp. HS66 Nat prod bioprospect 10(4):201–207
41. Zhao Z, Ding W, Wang PM, Zheng D, Xu J (2021) Five polyketides isolated from the marine-derived fungus *Arthrinium* Sp. Nat Prod Res 35(15):2470–2475
42. Yan X, Wen Y, Hu M, Wu Z, Tian X (2021) Promotion of the hypocrellin yield by a co-culture of *Shiraia bambusicola* (GDMCC 60438) with *Arthrinium* sp. AF-5 fungus. Fermentation 7(4): 316
43. Fu Y, Yin ZH, Wu LP, Yin CR (2016) Biotransformation of ginsenoside Rb1 to ginsenoside C-K by endophytic fungus *Arthrinium* sp. GE 17-18 isolated from Panax ginseng. Lett Appl Microbiol 63(3):196–201
44. Zhang Y, Zhang Q, Bao J, Huang J, Zhang H (2019) Apiosporamide, a 4-hydroxy-2-pyridone alkaloid, induces apoptosis via PI3K/Akt signaling pathway in osteosarcoma cells. Onco Targets Ther 12:8611
45. Li S, Tang Y, Fang X, Qiao T, Han S, Zhu T (2020) Whole-genome sequence of *Arthrinium phaeospermum*, a globally distributed pathogenic fungus. Genomics 112(1):919–929
46. Majeedano AQ, Chen J, Zhu T, Li S, Gishkori ZGN, Mastoi SM, Wang G (2022) The first whole genome sequence discovery of the devastating fungus *Arthrinium rasikravindrae*. J Fungi 8(3):255
47. Ramm S, Krawczyk B, Mühlenweg A, Poch A, Mösker E, Süssmuth RD (2017) A self-sacrificing n-methyltransferase is the precursor of the fungal natural product omphalotin. Angew Chem Int Ed 56:9994–9997
48. Xia X, Shim SH (2016) Secondary metabolites produced by endophytic fungus, Arthrinium phaeospermum. Korean J Pharmacogn 47(3):217–221

Wide Range Applications of Fungal Pigments in Textile Dyeing

10

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Abstract

The textile industry makes extensive use of synthetic, non-renewable colors. However, using these synthetic pigments causes the release of toxic and hazardous substances into the environment. It is a difficult task to find new pigments made from natural materials, yet doing so opens up safer textile coloring methods. A lot of attention has been paid to microbial pigments lately, particularly fungal pigments because of how safe and inexpensive they are. The sustainable manufacture and biodegradable properties of fungi-derived pigments are further benefits of their use. Fungal pigments have the potential to be used in a variety of applications, including textile colorants. A potential remedy for the hazardous side effects of synthetic pigments will be the industrial application of novel biotechnological techniques for the synthesis of fungal pigmentation. Carotenoids, flavins, indigo, melanins, monastics, phenazines, quinones, and violacein are some of the most promising fungal pigments. The numerous fungal pigments and how they are used to dye textile fibers are covered in this chapter. This chapter also emphasizes how fungal pigments will likely be used in a variety of biotechnological applications in the future.

Keywords

Fungal pigments · Natural pigments · Biotechnological applications · Industrial applications

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10.1 Introduction

A dye is a colored substance that adheres well to the surface it is applied. Natural colors are mostly derived from plant sources, including roots, berries, bark, leaves, and wood, as well as from animals and microbes. Natural dyes are less harmful to health, nontoxic, and non-polluting. Their antioxidant and antimicrobial properties also enhance their beneficial effects. The major goal of extracting colors from natural sources is to prevent the environmental risks that come with synthetic dyes, as well as the harmful and allergic reactions they might cause. These natural pigments have become a significant alternative to synthetic dyes.

Numerous stable colors produced by microbes, including anthraquinones, carotenoids, flavonoids, quinines, and rubramines, are well-known (Fig. 10.1). As they create stable colorants, fungi are a more fascinating and environmentally friendly source of pigments. Numerous anthraquinone molecules, as well as pigments like delphinine, melanin, and volatile chemical compounds, which have been referred to as their secondary metabolites, are produced by fungi [1]. Eco-friendly and nontoxic dyes are increasingly in demand, particularly for textiles for children and food products. Microbes that produce natural colors are a feasible alternative to toxic synthetic dyes and natural vegetable dyes. Thus, a

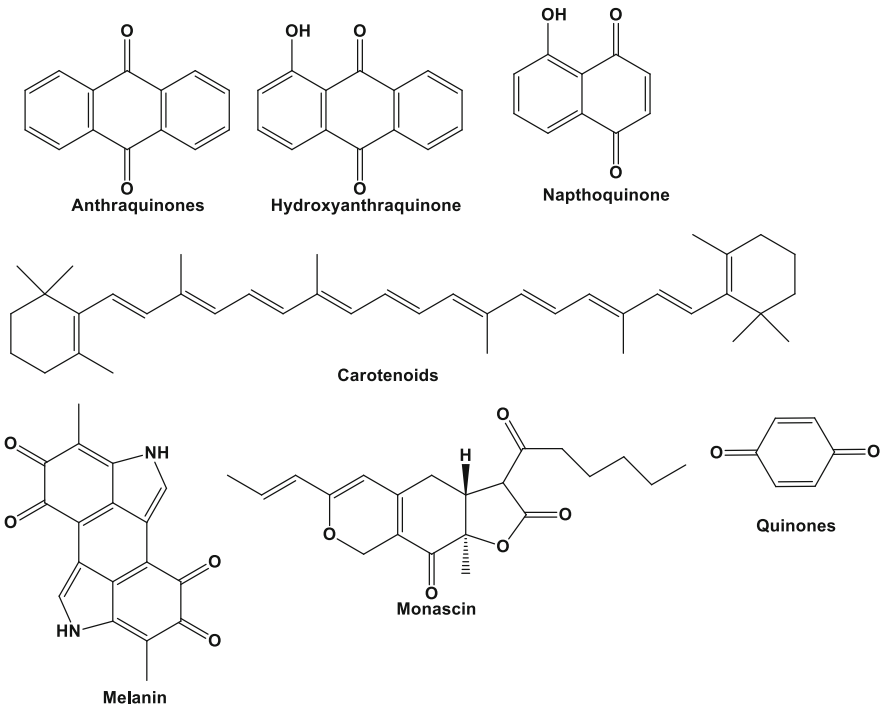

Fig. 10.1 Structures of some popular fungal pigments

possible biological source of natural colors like anthraquinones is claimed to be fungi [2].

Fungi are superior to plants as a source of pigments because they produce pigments all year round. In addition, fungi may grow more quickly and easily than plants, and they can be cultivated on less expensive culture media. In addition, it is possible to create pigments with a wide range of color hues and good solubility, stability, and hence processing ease [3–5]. Numerous fungi including *Alternaria alternata*, *Aspergillus niger*, *Curvularia lunata*, *Hapalopilus nidulans*, *Boletopsis grisea*, *Hypomyces lactifluorum*, *Trichoderma virens*, *Monascus purpureus*, *Isaria farinosa*, *Emericella nidulans*, *Fusarium verticillioides*, and *Penicillium purpurogenum* produce different shades of dyes and pigments as their intermediate metabolites [1]. In addition to the fungal pigments' commercial use in the painting and textile industries, fungal pigments are also used in a variety of other industries, including the creation of red mold rice by *Monascus* pigments, which are thought to be the earliest fungal pigment used by humans [6]. A prospective, promising food colorant and food additive is the red pigment produced by *M. ruber* [7]. *Penicillium oxalicum* produces the pink-red *Monascus* pigments, *Ashbya gossypii* provides the riboflavin, and *Blakeslea trispora* secretes the β-carotene which are some examples of fungal pigments.

As food colorants, fungi have previously entered and are still present on the market [8–10]. The usage of some fungal pigments as bioactive pigments with antimicrobial properties, on the other hand, is a wonderful application for them. *Monascus*, *Fusarium*, *Alternaria*, *Talaromyces*, *Trichoderma*, *Thermomyces*, *Penicillium*, *and Aspergillus* species' pigments exhibit antimicrobial properties against a variety of pathogenic bacteria, fungi, and yeast. Due to these properties, the pharmaceutical and food sectors nominate these pigments for use as food preservatives and antibacterial components (bandages, suture threads, and face masks) [11–19]. Similar to this, some fungal isolates, such as *F. oxysporum*, *T. verruculosus*, and *Chaetomium* spp., showed cytotoxic properties in their fungal pigments. Such activities have been evaluated using a variety of methods, including sour orange seed cell counting kit-8 (CCK-8) assay, brine shrimp lethality bioassay, or toxicity assay utilizing *Saccharomyces cerevisiae* [2, 20].

In the beauty industry, fungus pigments can also satisfy consumers' demands for natural alternatives to synthetic colors. Melanin, carotenoids, and lycopene, among other fungal pigments, have been used in a variety of products, including lipsticks, anti-aging facials, face creams, skin conditioners, and sunscreens [8, 21]. *Thermomyces* spp. and *P. purpurogenum* pigments were harmless to Wistar rats, which raised the possibility of their use in cosmetics and dyeing [22]. Several fungus pigments, including carotenoids, violacein, and naphthoquinones, have antioxidant properties. *Sanghuangporus baumii*, *Stemphylium lycopersici*, *Penicillium miczynskii*, *P. purpurogenum*, *P. purpurascens*, *Fusarium* sp., *Thermomyces* sp., *Chaetomium* sp., *Trichoderma afroharzianum*, and other *Trichoderma* spp. pigments are promising antioxidants that might be applied in the medical field [23–27].

Numerous fungal pigments are also known to have anticancer and antitumor properties. *Monascus purpureus* and *M. pilosus* produce pigments like monascin, ankaflavin, monaphilone A–B, monasphilone A–C, monapilol A–D, and monapurone A–C that have been shown to have anticancer/antitumor potential against various cancers, including mouse skin carcinoma, human laryngeal carcinoma, human hepatocellular carcinoma, human colon adenocarcinoma, and pulmonary adenocarcinoma [28, 29]. Additionally, benzoquinone from *Fusarium* spp., norsolorinic acid from *Aspergillus nidulans*, shiraiarin from *Shiraia bambusicola*, and alterporriol K, alterporriol L, and alterporriol M from *Alternaria* spp., all exhibit anticancer, antitumor, or antiproliferative activity, particularly against human breast cancer cell lines (MCF-7, MDA-MB-435, and MCF-7 b). Also, hypocrellin D produced by *Shiraia bambusicola* shows anticancer activity against Bel-7721, A-549, and Anip-973 cancer cell lines [30–33].

10.2 Natural Pigments

A significant substitute for possibly dangerous synthetic colors is a natural pigment. Due to many factors, including the industry's suffering from the high cost of raw materials for eco-friendly pigments, the door has recently opened for alternative pigment products made from natural sources. On the other hand, the problems with synthetic food colorants, which are known to cause nutrient loss in food, cause many negative effects on consumers' health, most notably types of cancer, abrupt mood swings, hyperactivity in children, DNA damage or genotoxicity, and attention-deficit disorder; this is in addition to their negative effects on the environment [34]. Some naturally occurring red pigments, such as the plant-derived anthocyanins, cannot withstand high pH. Other colorants derived from plants or animals are not available all year and become unstable in the presence of heat and light [35]. As a result, there is a pressing need for microbiological natural colorants. According to reports, some microorganisms, including *Paecilomyces*, *Monascus*, *Cordyceps*, and *Penicillium* spp., generate colorants [36].

Due to the rapid growth of microorganisms, they can produce more microbial colorants than nontoxic eco-friendly substances [37]. A wide range of colors that are water-soluble is produced by microorganisms. The genus *Penicillium* is said to produce high-quality polyketide pigments [38]. One of the most well-known industrial sectors in the world is the textile industry. This sector has been identified as one of the most polluting industries because it releases effluents that are contaminated with numerous pollutants, the most dangerous of which are colorants, which are also non-biodegradable [39].

The two main natural protein fibers used in the textile industry—wool and silk—have a sizable market share worldwide. They can be colored with acid and reactive dyestuffs due to the nature of their acidic and basic niches. Due to their capacity to create covalent bonds with wool and silk, sensitive dyes are typically utilized as colorants for both substrates [40]. On the global market, artificial colorants are available in a wide range of variety and at affordable prices. However, there are

numerous issues with their use in textile coloring, including skin allergies and irritation, hazardous chemical release during their synthesis, and, in certain instances, carcinogenic effects.

As a result, research on textile colorants has focused on finding alternatives to synthetic pigments. Microbial colorants are now in high demand on the market. Many researchers have used microbes to produce colorants by fungi like *Monascus purpureus*, *Penicillium purpurogenum*, *Paecilomyces sinclairii*, *Cordyceps unilateralis*, and others [41–43].

10.3 Ecology of Fungal Pigments

All around the world, eukaryotic fungi are numerous and diverse. Fungi can be found in many different ecosystems from terrestrial areas to marine surroundings. Additionally, they can be found in a variety of habitats with a wide range of temperatures, from polar to tropical areas. There is a wide diversity of fungi that can be found, even in harsh conditions. When considered collectively, these circumstances enable fungi to create a wide range of new secondary metabolites [24]. Recent research has shown that fungal sources, including new pigments from marine settings, can produce a variety of unique compounds [44]. Marine microbial ecology has not been well-studied. More research is required to characterize marine microorganisms and their new metabolites because marine microbial ecology has not been adequately studied. On the other hand, due to the availability of various organic substances that encourage the development of numerous secondary metabolites, tropical ecosystems offer possible habitats for a diversity of fungi. According to earlier research, pigmented fungi are more tolerant to intense radiation and hydration/dehydration cycles than non-pigmented fungi. For instance, the melanin generated by some filamentous fungi has antioxidant properties that shield fungal structures and aid in survival in hostile conditions [45].

10.4 Fungal Pigments of Commercial Importance

Filamentous fungi have demonstrated the ability to produce a variety of incredible colors. Since the majority of these fungal pigments are water-soluble, they have found use in numerous commercial sectors, most notably the textile dyeing industry. The simplicity of their scale-up manufacture in industrial fermenters and their extraction without organic solvents are further benefits for the fungal pigments. Synthetic colors are not biodegradable and non-renewable, and they pollute the environment with harmful waste. Because of this, disposing of the waste from the by-products of synthetic colors presents a hurdle. It is more cost-effective to produce microbial pigments using fermentation technology, which yields biodegradable substances with commercial uses as colorants [46, 47].

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Table 10.1 Fungal pigments and their application in the textile industry

Fungi	Pigment	Color	Fabrics	References
Alternaria alternata	Anthraquinones	Reddish-brown	Cotton	Devi and Karuppan [50]
Penicillium oxalicum	Anthraquinones	Arpink red	Wool	Sardaryan et al. [51]
Chlorociboria aeruginosa *Scytalidium cuboideum* *Scytalidium ganodermophthorum*	Quinones	Green Red Yellow	Bleached cotton, spun polyacrylic, spun polyamide, spun polyester, worsted wool	Weber et al. [49]
Aspergillus sp.	Quinones	Brown cotton	Silk, silk cotton	Devi and Karuppan [50]
Acrostalagmus (NRC 90) *Alternaria alternata* (NRC17) *Alternaria* sp. (NRC 97) *Aspergillus niger* (NRC 95), *Bisporomyces* sp. (NRC 63)	Quinones	Brown Reddish-brown Brown Brown Deep brown	Wool	Atalla et al. [52]
Penicillium murcianum	Carotenoids	Yellow	Wool	Hernández et al. [53]
Talaromyces australis	2, 4-Di-tert-butylphenol	Red	Cotton fabric	Shibila and Nanthini [54]
Phoma herbarum	Magenta pigment	Magenta	Nylon	Chiba et al. [55]
Talaromyces verruculosus	Polyketide	Red	Cotton fabric	Chadni et al. [56]
Monascus purpureus	Rubropunctamine	Red	Wool	De Santis et al. [57]

Carotenoids, flavins, melanins, monascins, phenazines, and quinones are a few examples of the commercially significant pigments that fungi generate [48]. Due to their nontoxicity and environmental friendliness, many fungal pigments have acquired popularity. For instance, *Chlorociboria aeruginosa*, *Scytalidium ganodermophthorum*, and *Scytalidium cuboideum* were used to identify three eco-friendly fungal pigments with the ability to dye textiles without the use of water or heat [49]. Over a duration of 1 week, the pigments exhibited no fading. However, further research is needed to comprehend how the colors interact with other elements like materials, stability (time, UV, wash and wear), skin sensitivity, and toxicity. Table 10.1 displays how fungi and their pigments are applied in the textile industry.

10.5 Fungal Dyes for Textile Applications Including Cotton, Wool, and Silk Fabrics

One of the sectors with the greatest impact on the economy and employment is the textile industry. Fungal pigments are extremely valuable in the textile industry, especially cotton and wool dyeing, due to their distinctive staining characteristics. The generation of biodegradable fungi pigments can be easily managed by modifying the necessary circumstances such that there are no seasonal variations [57]. However, due to inconsistent fixation and a lack of regulated procedures for applying fungal pigments in the textile industry, their qualities still fall short of the standards demanded by the sector [49, 58, 59]. It's crucial to develop a suitable procedure for standardizing the use of fungal dyes in the sector. The textile industry has employed fungi pigments as a substitute for several hazardous or toxic synthetic colors due to their uniformity and stability [60]. The application of fungal pigments to fabrics is reported to absorb light in the ultraviolet area, shielding the skin from harmful UV radiation [61]. The potential of fungi-based pigments in the textile industry has been covered in many research [21, 62]. Fungi such as *Aspergillus* sp., *Curvularia* sp., *Drechslera* sp., and *Trichoderma* sp. are known to produce anthraquinones. Cynodontin was extracted from *Curvularia lunata*, and it produces two anthraquinone dyes which are similar to both Acid green 28 and Disperse blue 7 [63]. It is interesting to note that anthraquinones produced by several fungi have been found to exhibit antibacterial properties. Additionally, *Sclerotinia* sp. produces anthraquinones which have a pink hue and are used to color cotton yarns. The yarns have shown promising resistance to a variety of environments, including heat, light, and different pH levels [64].

Alternaria alternata produced reddish-brown pigments, according to Devi and Karuppan [50]. These pigments demonstrated great colorfastness against rubbing and perspiration as well as efficiency in coloring cotton materials [50]. Velmurugan et al. evaluated other water-soluble fungal pigments produced by *Emericella nidulans*, *Fusarium verticillioides*, *Isaria farinosa*, *Monascus purpureus*, *and Penicillium purpurogenum*, and these pigments were employed to color cotton fibers [65]. Numerous fungal pigments have been used to color wool such as water-soluble yellow and red pigments which are produced by *P. murcianum* and where the red dyes resemble pigments of the *Monascus* class [66, 67]. These two dyes have been discovered to have a preference for wool, and they have demonstrated durability under a variety of industrial circumstances since they can survive changes in temperature and pH. Table 10.2 contains a list of industrially manufactured and used fungi pigments.

According to another study, *Penicillium* sp. produces ankaflavin (pigments of the *Monascus* type), which has a great affinity for wool due to its ionic nature [38, 68] (Fig. 10.2). Figure 10.3 illustrates how the response of the pigment made by *Penicillium purpurogenum* changes according to the materials tested. While viscose and polyester failed to absorb the dye, wool and silk absorbed dye well [38].

Anthraquinones derived from *Fusarium oxysporum* also showed excellent color fastness and high dye uptake, making them an ideal dye for wool

Table 10.2 List of fungal pigments produced on an industrial scale

Fungal species	Pigments	References
Monascus species	Ankaflavin (yellow), monascorubramine (red), rubropunctatin (orange)	Caro et al. [8]
Ophiocordyceps unilateralis	Erythrostominone (red), 3,5,8-TMON (red)	Caro et al. [8]
Blakeslea trispora	β-Carotene (yellow-orange), lycopene (red)	Caro et al. [8]
Ashbya gossypii	Riboflavin (yellow)	Caro et al. [8]
Penicillium oxalicum	Anthraquinone derivative (red), anthraquinones (red and other hues) Arpink red™, secalonic acid D (yellow)	Caro et al. [8]

Fig. 10.2 Different factors for optimization of red pigment production by *Penicillium purpurogenum* (These photos were taken by Dr. Waill A. Elkhateeb)

[69]. Anthraquinones also have the benefit of producing bright hue colors, and it is possible to modify them chemically to make the manufacturing of dye molecules simpler. According to Morales-Oyervides et al., *Talaromyces* sp. pigment has a utility in wool fabrics. In addition to demonstrating a high pigment uptake by wool

Fig. 10.3 Effect of red pigment produced by *Penicillium purpurogenum* application on different fabrics (wool, silk, polyester, viscose). (This photo was taken by Dr. Waill A. Elkhateeb)

fabric, this study also demonstrated constant dyeing rates, half-time dyeing, and kinetic behaviors resembling those of natural dyes [70].

Trichoderma sp. was used to produce a pigment that Gupta et al. extracted and examined for application in dyeing both silk and wool fibers. According to the findings, the *Trichoderma* sp. dye was safe to human skin; moreover, it demonstrated antimicrobial characteristics. Additionally, the findings indicated that due to wool's superior fastness characteristics against rubbing and washing, it has a better affinity for dyeing than silk cloth [71]. *Talaromyces* pigment, another fungus with antimicrobial qualities, is nontoxic and might be employed as a substitute for the natural pigments used in the textile industry [55]. But more analysis of the interplay between dye and fabric is needed. According to Hinsch et al., several fungus pigments were recovered from decaying Canadian hardwood logs. These pigments included yellow pigment (from *Scytalidium ganodermophthorum*), draconin red (red pigment from *Scytalidium cuboideum*), and xylindein (green pigment obtained from *Chlorociboria aeruginosa*). Without the use of extra chemicals, the three pigments colored test strips for multiple fabrics [72]. Among these pigments, xylindein showed a good capacity to color textiles for clothing. Researchers have proposed using natural oils to transport colors from the three fungi (*Chlorociboria* sp., *Scytalidium cuboideum*, and *Scytalidium ganodermophthorum*) onto host substrate [73]. Applying these organic oils would help prevent environmental damage and other health problems that come with utilizing the hazardous dichloromethane (DCM) to extract colorants, which is one of the main reasons why the commercialization of fungus-based pigments has been restricted [73]. A yellow pigment that has been utilized in textile dyeing was also discovered to be produced by *Thermomyces* species. The yellow pigment together with the natural mordants was found to inhibit the growth of many pathogens. Thus, this yellow pigment has

been employed in several medicinal applications, including bandages, masks, and wound dressings [74].

10.6 Biotechnological Approaches for Improving Fungal Pigment Production

Enhancing fungal pigment production and improving their yield have been achieved through genetic manipulation, where the pathways for pigment production and the various molecules included will be better understood. Also, molecular screening techniques can improve gene expression resulting in the production of novel metabolites. Additionally, pigment production can be enhanced via the optimization of the fermentation conditions for pigment production. It is difficult to use different microorganisms for pigment production at the commercial level today because of the increased industrial need for pigments (Fig. 10.4).

Enhancing the production of fungal pigment requires the use of genetic engineering techniques such as gene cloning, genetic modification, and elimination of non-essential genes. For instance, *Phycomyces blakesleeanus* and *Blakeslea trispora*, two natural carotenogenic fungi, are presently cultured up to an industrial scale where 17 grams of β-carotene may be obtained per liter. Additionally, by using

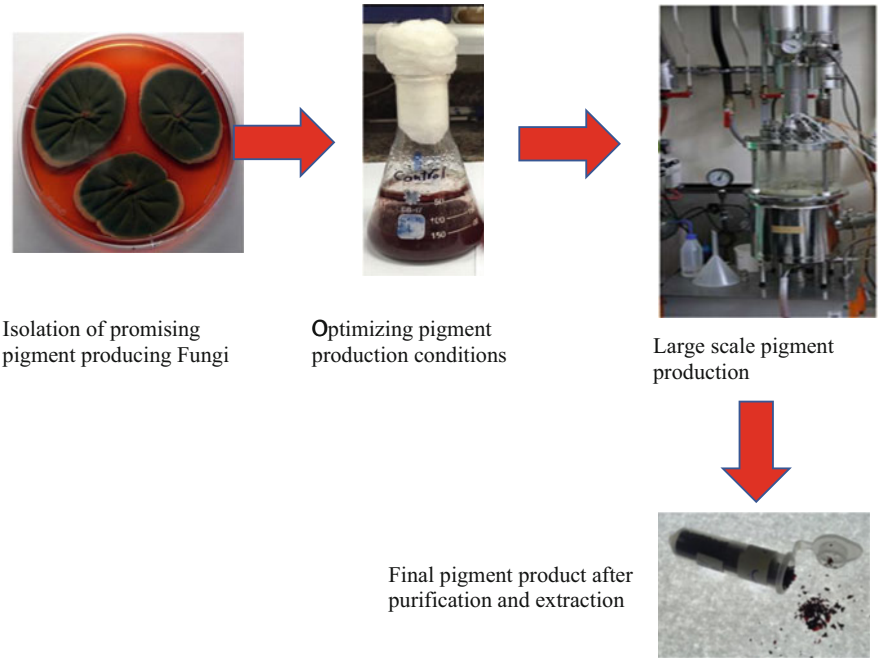

Fig. 10.4 Schematic diagram for the fungal pigment production from lab scale to industrial scale. (These photos were taken by Dr. Waill A. Elkhateeb)

metabolic engineering approaches, numerous efforts have been conducted to establish the β-carotene biosynthetic pathway in non-carotenogenic strains. The studies showed that enhancing the dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) precursors of the MVA or MEP pathways improved the production of β-carotene and lycopene [75]. Fungal strains that generate polyketides are another illustration. Due to their dense and filamentous mycelial development, which raises the viscosity of the culture medium and reduces oxygen transport, these strains are difficult to cultivate in liquid fermentations. The fermentation procedure was made simpler by transferring the polyketide biosynthesis pathway using metabolic engineering techniques into an industrial strain of *Saccharomyces cerevisiae* that does not display dense filamentous growth [76]. As previously indicated, improving the fermentation conditions has a significant impact on the generation of secondary metabolites. Three pigments, citrinin, monacolin K, and red pigment, are produced by the *Monascus* genus of mold [77]. Numerous methods have been used to increase the production of the red pigment by reducing the formation of citrinin (mycotoxin). To reduce citrinin production, numerous parameters have been optimized, including temperature, pH, dissolved oxygen, carbon and nitrogen sources, and other genetic changes. Disruption of the polyketide synthase gene *pksCT* has successfully eliminated citrinin production in the industrial strain, *Monascus purpureus* SM001 [78].

10.7 Challenges and Prospects for the Industrial Application of Fungal Pigments

Because many synthetic textile dyes contain dioxins (polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans), which are known to cause cancer, researchers have been looking into safer, nontoxic alternatives. Fungal pigments offer a potential remedy for this issue. The customary and regional usage and the permitted applications of fungal pigments (J. Fungi 2020, 6, 68; 16 of 23) vary by country [48]. For instance, Mapari et al. [76] have received patents for the uses of *Monascus*-like pigments derived from *Penicillium* sp. on cotton, wool, textile, leather, etc. Additionally, certain efforts have been made to enhance the manufacturing of pigments as well as their stability and water solubility [76]. By promoting fungal growth and increasing the pigment's accumulation inside cells, the production of this pigment has been boosted [79]. The challenge of raising both biomass output and pigment because they are negatively connected is the key issue that has yet to be solved. The findings show that genetic engineering methods can be used to regulate pigment production. The activity of the enzymes involved in the synthesis of carotenoids has been altered using recombinant DNA methods.

The downsides of *Monascus* pigment are its poor resilience to heat, light, and pH, as well as its limited water solubility. This made it difficult to produce fungal pigments for use in industry. Dufossé's study was used to overcome this problem by replacing oxygen with nitrogen from the amino group that exists in the pigment structure [9]. Another main problem faced in the commercial use of fungal pigments

is the co-production of toxic compounds together with the pigment. Most of the fungal pigments are produced in mixtures with other unfavorable compounds that may affect the pigment tone and toxicity. In this case, the challenge is to produce a single pigment product with one specific color tone. This can be done by adjusting the fermentation conditions, including the substrates, temperature, pH, and dissolved oxygen, whether in submerged fermentation or solid-state. Since many fungal metabolites are limited for their commercialization due to the production of mycotoxins together with pigments, toxicological tests are needed to ensure the final applications of the pigments in different products such as food, cosmetics, textiles, etc. There is also a need to develop fungal pigments at the industrial level by choosing suitable strains with safety measures. More attention should be directed to producing cost-effective fungal pigments on an industrial scale. Another important point is pigment stability over time and under various conditions.

The use of microbes for pigment production has advantages since it does not need petroleum-based raw materials which affects the pigment price. To meet the current demand for natural pigments, it is necessary to find more unique pigment-producing fungi from various taxonomic groupings. Therefore, additional qualitative and quantitative study on fungi with detrimental ecological effects is needed. To be accepted by customers, toxicological testing of highly potent fungal pigments must be completed.

10.8 Conclusions

Naturally synthesized pigments are important bioactive compounds that have a high level of market demand. Many scientists were interested in discovering natural dyes that were nontoxic and eco-friendly that came from renewable sources. Fungal pigments' potential for industrial uses, particularly in textiles, has recently come under investigation. Numerous fungi have demonstrated their capacity to manufacture a variety of harmless and biodegradable colors. Additionally, these fungal pigments have color stability that endures a range of environmental conditions, including varying temperatures, pH levels, washing, and wearing. The quantity and quality of fungal pigments have been improved using a variety of techniques in genetic engineering and biotechnology. However, more research is called for reducing toxicity and improving the quality of these colors to match commercial needs and gain approval to enter markets.

References

1. Aishwarya AD (2014) Extraction of natural dyes from fungus – an alternate for textile dyeing. J Nat Sci Res 4(7):1–6
2. Sayyed I, Majumder D (2015) Pigment production from fungi. Int J Curr Microbiol App Sci Special Issue-2:103–109
3. Joshi VK, Attri D et al (2003) Microbial pigments. Indian. J Biotechnol 2:362–369

4. Lagashetti AC, Dufossé L et al (2019) Fungal pigments and their prospects in different industries. Microorganisms 7(12):604
5. Manikprabhu D, Lingappa K (2013) γ Actinorhodin a natural and attorney source for the synthetic dye to detect acid production of fungi. Saudi J Biol Sci 20:163–168
6. Went FA (1895) Monascus purpureus le champignon de l'ang-quac une nouvelle thelebolee. Ann Des Sci Nat Bot Biol Veg 8:1–18
7. Fabre CE, Santerre AL et al (1993) Production and food applications of the red pigments of Monascus ruber. J Food Sci 58:1099–1102
8. Caro Y, Venkatachalam M et al (2017) Pigments and colorants from filamentous fungi. In: Merillon J-M, Ramawat KG (eds) Fungal metabolites. Springer International Publishing, Cham, pp 499–568
9. Dufossé L (2006) Microbial production of food grade pigments. Food Technol Biotechnol 44: 313–323
10. Kim D, Ku S (2018) Beneficial effects of Monascus sp. KCCM 10093 pigments and derivatives: a mini review. Molecules 23:98
11. Frandsen RJ, Rasmussen SA et al (2016) Black perithecial pigmentation in fusarium species is due to the accumulation of 5-deoxybostrycoidin-based melanin. Sci Rep 6:26206
12. Manon Mani V, Shanmuga Priya M et al (2015) Antioxidant and antimicrobial evaluation of bioactive pigment from fusarium sp. isolated from the stressed environment. Int J Curr Microbiol Appl Sci 4:1147–1158
13. Martinkova L, Juzlova P et al (1995) Biological activity of polyketide pigments produced by the fungus Monascus. J Appl Bacteriol 79:609–616
14. Patil SA, Sivanandhan G et al (2015) Effect of physical and chemical parameters on the production of red exopigment from *Penicillium purpurogenum* isolated from spoilt onion and study of its antimicrobial activity. Int J Curr Microbiol Appl Sci 4:599–609
15. Saravanan D, Radhakrishnan M (2016) Antimicrobial activity of pigments produced by fungi from the Western Ghats. J Chem Pharm Res 8:634–638
16. Sibero MT, Triningsih DW et al (2016) Evaluation of antimicrobial activity and identification of yellow pigmented marine sponge-associated fungi from Teluk Awur, Jepara, Central Java. Indones J Biotechnol 21:1–11
17. Suryanarayanan TS, Ravishankar JP et al (2004) Characterization of the melanin pigment of a cosmopolitan fungal endophyte. Mycol Res 108:974–978
18. Vendruscolo F, Tosin I et al (2014) Antimicrobial activity of *Monascus* pigments produced in submerged fermentation. J Food Process Preserv 38:1860–1865
19. Yolmeh M, Hamedi H et al (2016) Antimicrobial activity of pigments extracted from Rhodotorula glutinis against some bacteria and fungi. Zahedan J Res Med Sci 18:e4954
20. Malik K, Tokas J et al (2016) Characterization and cytotoxicity assay of pigment-producing microbes. Int J Curr Microbiol Appl Sci 5:370–376
21. Sajid S, Akbar N (2018) 1. Applications of fungal pigments in biotechnology. Pure Applied Biology (PAB) 7:922–930
22. Poorniammal R, Prabhu S et al (2019) Evaluation of in vitro antioxidant activity of fungal pigments. Pharma Innov J 8:326–330
23. Li F, Xue F et al (2017) FTIR and Raman analysis of antioxidant components of red pigments from Stemphylium lycopersici. Curr Microbiol 74:532–539
24. Ramesh C, Vinithkumar NV et al (2019) Multifaceted applications of microbial pigments: current knowledge, challenges and future directions for public health implications. Microorganisms 7:186
25. Sen T, Barrow CJ, Deshmukh SK (2019) Microbial pigments in the food industry challenges and the way forward. Front Nutr 6:7
26. Tuli HS, Chaudhary P et al (2015) Microbial pigments as natural color sources: current trends and future perspectives. J Food Sci Technol 52:4669–4678
27. Vendruscolo F, Buhler RM et al (2016) Monascus: a reality on the production and application of microbial pigments. J Appl Biochem Biotechnol 178:211–223

28. Akihisa T, Tokuda H et al (2005) Anti-tumor-initiating effects of Monascin, an azaphilonoid pigment from the extract of Monascus pilosus fermented rice (red-mold rice). Chem Biodivers 2:1305–1309
29. Su N-W, Lin Y-L et al (2005) Ankaflavin from Monascus-fermented red rice exhibits selective cytotoxic effect and induces cell death on Hep G2 cells. J Agric Food Chem 53:1949–1954
30. Cai Y, Ding Y et al (2008) Production of 1, 5-dihydroxy-3-methoxy-7-methylanthracene-9, 10-dione by submerged culture of Shiraia bambusicola. J Microbiol Biotechnol 18:322–327
31. Fang LZ, Qing C et al (2006) Hypocrellin D, a cytotoxic fungal pigment from fruiting bodies of the ascomycete Shiraia bambusicola. J Antibiot 59:351–354
32. Feng Y, Shao Y et al (2012) Monascus pigments. J. Appl Microbiol Biotechnol 96:1421–1440
33. Soumya K, Narasimha Murthy K et al (2018) Characterization of a red pigment from *Fusarium chlamydosporum* exhibiting selective cytotoxicity against human breast cancer MCF-7 cell lines. J Appl Microbiol 125:148–158
34. Shahid M, Mohammad F (2013) Recent advancements in natural dye applications: a review. J Clean Prod 53:310–331
35. Gunasekaran S, Poorniammal R (2008) Optimization of fermentation conditions for red pigment production from *Penicillium* sp. under submerged cultivation. Afr J Biotechnol 7(12): 1–12
36. Suhr KI, Haasum I et al (2002) Factors affecting growth and pigmentation of *Penicillium caseifulvum*. J Dairy Sci 85(11):2786–2794
37. Méndez-Zavala A, Contreras-Esquivel JC et al (2007) Fungal production of the red pigment using a xerophilic strain *Penicillium purpurogenum* GH-2. Revista mexicana de ingeniería química 6(3):267–273
38. Ahmed EF, Elkhateeb WA et al (2018) Wool and silk fabrics dyeing by mannitol-assisted pigment produced from *Penicillium purpurogenum*. Der Pharma Chemica 10(10):166–175
39. Khan S, Malik A (2014) Environmental and health effects of textile industry wastewater. In: Environmental deterioration and human health. Springer, Dordrecht, pp 55–71
40. Hernández-Rivera J, Méndez-Zavala A et al (2008) Culture conditions to improve the red pigment production by *Penicillium purpurogenum* GH2. In: Advance in food science and food biotechnology in developing countries. Mex Asoc Food Sci Editions, Saltillo, pp 108–112
41. Elkhateeb WA, Morsi SS et al (2020) Immobilization of *Penicillium purpurogenum* and application of the produced pigment in paint industry. Plant Arch 20(1):857–862
42. Jiang Y, Li HB et al (2005) Production potential of water-soluble *Monascus* red pigment by a newly isolated *Penicillium* sp. J Agric Technol 1(1):113–126
43. Unagul P, Wongsa P et al (2005) Production of red pigments by the insect pathogenic fungus *Cordyceps unilateralis* BCC 1869. J Ind Microbiol Biotechnol 32(4):135–140
44. Zain ul Arifeen M, Ma Y-N et al (2020) Deep-sea fungi could be the new arsenal for bioactive molecules. Mar Drugs 18:1–9
45. Eisenman HC, Casadevall A (2012) Synthesis and assembly of fungal melanin. Appl Microbiol Biotechnol 93:931–940
46. Silva TR, Tavares RS et al (2019) Chemical characterization and biotechnological applicability of pigments isolated from Antarctic bacteria. Mar Biotechnol 21:416–429
47. Venil CK, Devi PR et al (2020) Agro-industrial waste as substrates for the production of bacterial pigment. In: Valorisation of Agro-industrial Residues–Volume I: Biological Approaches. Springer, pp 149–162
48. Dufosse L, Fouillaud M et al (2014) Filamentous fungi are large-scale producers of pigments and colorants for the food industry. Curr Opin Biotechnol 26:56–61
49. Weber G, Chen HL et al (2014) Pigments extracted from the wood-staining fungi *Chlorociboria aeruginosa*, *Scytalidium cuboideum*, and *S. ganodermophthorum* show potential for use as textile dyes. Color Technol 130:445–452
50. Devi S, Karuppan P (2015) Reddish brown pigments from *Alternaria alternata* for textile dyeing and printing. Ind J Fibre Textile Res 40(3):315–319

51. Sardaryan E, Zihlova H et al (2004) Arpink red–meet a new natural red food colorant of microbial origin. In: Pigments in food, more than colours, pp 207–208
52. Atalla MM, Elkhrisy E et al (2011) Production of textile reddish brown dyes by fungi. Malaysian J Microbiol 7:33–40
53. Hernández VA, Galleguillos F et al (2019) Fungal dyes for textile applications: testing of industrial conditions for wool fabrics dyeing. J Textile Institute 110:61–66
54. Shibila S, Nanthini A (2019) Extraction and characterization of red pigment from Talaromyces australis and its application in dyeing cotton yarn. Int Arch App Sci Technol 10:81–91
55. Chiba S, Tsuyoshi N et al (2006) Magenta pigment produced by fungus. J Gen Appl Microbiol 52:201–207
56. Chadni Z, Rahaman MH et al (2017) Extraction and optimisation of red pigment production as secondary metabolites from *Talaromyces verruculosus* and its potential use in textile industries. Mycology 8:48–57
57. De Santis D, Moresi M et al (2005) Assessment of the dyeing properties of pigments from Monascus purpureus. J Chem Technol Biotechnol Int Res Proc Environ Clean Technol 80: 1072–1079
58. Hinsch EM, Robinson SC (2016) Mechanical color reading of wood-staining fungal pigment textile dyes: an alternative method for determining colorfastness. Coatings 6:25
59. Sharma D, Gupta C et al (2012) Pigment extraction from fungus for textile dyeing. Ind J Fibre Textile Res 37(1):68–73
60. Robinson SC, Tudor D et al (2012) Utilizing pigment-producing fungi to add commercial value to American beech (Fagus grandifolia). Appl Microbiol Biotechnol 93:1041–1048
61. Nambela L, Haule LV et al (2020) A review on source, chemistry, green synthesis and application of textile colorants. J Clean Prod 246:119036
62. Kumar A, Vishwakarma HS et al (2015) Microbial pigments: production and their applications in various industries. Int J Pharmaceut Chem Biol Sci 5:1–10
63. Räisänen R (2009) Handbook of natural colorants. John Wiley & Sons, Chichester
64. Perumal K, Stalin V et al (2009) Extraction and characterization of pigment from *Sclerotinia* sp. and its use in dyeing cotton. Text Res J 79:1178–1187
65. Velmurugan P, Kim M-J et al (2010) Dyeing of cotton yarn with five water soluble fungal pigments obtained from five fungi. Fibers Polym 11:598–605
66. Osmanova N, Schultze W et al (2010) Azaphilones: a class of fungal metabolites with diverse biological activities. Phytochem Rev 9:315–342
67. Visagie C, Houbraken J et al (2014) Identification and nomenclature of the genus *Penicillium*. Stud Mycol 78:343–371
68. Afshari M, Shahidi F et al (2015) Investigating the influence of pH, temperature and agitation speed on yellow pigment production by *Penicillium aculeatum* ATCC 10409. Nat Prod Res 29: 1300–1306
69. Nagia F, El-Mohamedy R (2007) Dyeing of wool with natural anthraquinone dyes from *fusarium oxysporum*. Dyes Pigments 75:550–555
70. Morales-Oyervides L, Oliveira J et al (2017) Assessment of the dyeing properties of the pigments produced by *Talaromyces* spp. J Fungi 3:38
71. Gupta C, Sharma D et al (2013) Pigment production from *Trichoderma* spp. for dyeing of silk and wool. Int J Sci Nat 4:351–355
72. Hinsch EM, Weber G et al (2015) Colorfastness of extracted wood-staining fungal pigments on fabrics: a new potential for textile dyes. J Textile Apparel Technol Manag 9:1–10
73. Palomino Agurto ME, Vega Gutierrez SM et al (2017) Wood-rotting fungal pigments as colorant coatings on oil-based textile dyes. Coatings 7:152
74. Poorniammal R, Parthiban M et al (2013) Natural dye production from Thermomyces sp fungi for textile application. Ind J Fibre Textile Res 38(3):276–279
75. Rugbjerg P, Naesby M et al (2013) Reconstruction of the biosynthetic pathway for the core fungal polyketide scaffold rubrofusarin in *Saccharomyces cerevisiae*. Microb Cell Factories 12: 1–9

76. Mapari SA, Meyer AS et al (2009) Identification of potentially safe promising fungal cell factories for the production of polyketide natural food colorants using chemotaxonomic rationale. Microb Cell Factories 8:1–15
77. Jia XQ, Xu ZN et al (2010) Elimination of the mycotoxin citrinin production in the industrial important strain Monascus purpureus SM001. Metab Eng 12:1–7
78. Loto I, Gutiérrez MS et al (2012) Enhancement of carotenoid production by disrupting the C22-sterol desaturase gene (CYP61) in Xanthophyllomyces dendrorhous. BMC Microbiol 12: 1–16
79. Gmoser R, Ferreira JA et al (2017) Filamentous ascomycetes fungi as a source of natural pigments. Fung Biol Biotechnol 4:1–25

Part III

Fungi in Food Biotechnology

The Potential of Mushrooms in Developing Healthy Food and Biotech Products

11

S. M. Badalyan and A. Zambonelli

Abstract

Wild and cultivated edible and medicinal mushrooms have long been known by humans as a source of valuable food and medicines in Asian and European countries. Currently, only a small fraction of estimated fungal biodiversity has been investigated for their bioactivities and medicinal properties, while mushrooms possess a potential in pharmacy, medicine, cosmetics and food industry. In the kingdom of fungi, mushrooms taxonomically belong to phyla Basidiomycota (class Agaricomycetes) and Ascomycota (class Pezizomycetes) of the subkingdom Dikarya.

Mushrooms, such as truffles (*Tuber*), morels (*Morchella*), *Agaricus bisporus*, *Boletus edulis* and oyster mushrooms *(Pleurotus* species), are considered gourmet healthy food. Mushrooms (*Ganoderma* and *Trametes* species, *Hericium erinaceus*, *Lentinula edodes*, etc.) are also perspective sources for myco- pharmacological research as source of bioactive molecules (alkaloids, lipids, phenolics, polysaccharides, proteins, steroids, terpenoids, etc.) with more than 130 medicinal effects (anti-inflammatory, antimicrobial, antioxidant, antitumor, antiviral, cytotoxic, hepatoprotective, hypocholesterolaemic, hypoglycaemic, hypotensive, immunomodulatory, neuroprotective, etc.). There is scientific evidence of using mushroom-derived biotech products as dietary food, pharmaceuticals, cosmeceuticals and other products available in the market.

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The current review discusses recent advances in research on the biotechnological potential of mushrooms to develop novel biotech products and perspectives for their applications in human welfare.

Keywords

Biotech products · Cosmeceuticals · Medicinal · Mushrooms · Nutriceuticals · Pharmaceuticals

11.1 Introduction

Basidiomycota and Ascomycota fungi (classes Agaricomycetes and Pezizomycetes) of the subkingdom Dikarya, which develop epigeal and hypogeal fruiting bodies or mushrooms, are known to mankind not only as valuable gourmet foods, but also for their medicinal significance. They are producers of different bioactive compounds (polysaccharides, terpenoids, phenolics, polyketides, alkaloids, lectins, proteins, steroids, etc.) with potential pharmacological effects (anti-inflammatory, antioxidant, anti-proliferative, antiviral, hypocholesterolaemic, hypoglycaemic, hypotensive, immunomodulatory, neuroprotective, wound-healing, etc.) [1–34].

Mushrooms have been used by Eastern and Western civilisations since ancient times (Fig. 11.1). Among agaricomycete fungi medicinal *Ganoderma* species produce the highest diversity of pharmacologically active compounds [8, 27, 34–49].

Fig. 11.1 Covered vase decorated with female figure holding Lingzhi fungus (*Ganoderma lucidum*). China, Qing Dynasty, 18th century, Worcester Art Museum, Massachusetts (from Wikimedia)

The medicinal properties were reported in edible agaricomycete oyster mushrooms (*Pleurotus cornucopiae*, *P. djamor*, *P. eryngii*, *P. giganteus*, *P. ostreatus*, *P. levis*, *P. pulmonarius*, *P. sajor-caju*, *P. tuber-regium).* They are producers of diterpenoid eryngiolide A, polysaccharides and other biomolecules, particularly with hypoglycaemic, hypocholesterolaemic, neurotoxic and cardioprotective effects [5, 8, 9, 50–65].

Highly prized edible ascomycete hypogeal mushrooms are *Tuber* (the true truffles), *Terfezia* and *Tirmania* (the desert truffles) [66], as well as *Morchella* (true morels) species which develop epigeal ascomata [67–69]. These mushrooms are biotechnologically cultivated and have significant economic value due to their excellent gastronomic and medicinal properties [5, 17, 70–75].

Currently, the pathways of biosynthesis of bioactive molecules and the related genes are largely understudied. Recent data for identification of genes and gene clusters of bioactive molecules (terpenoids, phenolics, polyketides, cyclic peptides, aegerolysins, lectins, ribosome-inactivating proteins, etc.) in medicinal and edible mushrooms has been reviewed [27, 76]. Genes for pharmacologically active molecules are found in only a restricted number of fungal taxa and species. Some medicinal mushrooms probably have genes for a higher variety of bioactive compounds than species being commonly neglected for exploitation [27, 77–79].

Meanwhile, wild and cultivable, edible and medicinal mushrooms may be considered as valuable sources to develop different health-enhancing biotech products, such as pharmaceuticals, nutraceuticals and cosmeceuticals [10, 17, 71, 80]. Advances in biology and biotechnological cultivation of selected taxonomic groups of edible and medicinal mushrooms will further assist in the production of novel mushroom-derived biotech products for human welfare.

In this chapter, we have reviewed advancements on the bioactive compounds of wild and cultivated mushrooms, besides the new perspectives for exploiting them to produce new biotech products.

11.2 Biotechnologically Important Edible and Medicinal Agaricomycete and Ascomycete Mushrooms

In 2004, Boa [81] listed 2327 species of mushroom that were consumed or have medicinal properties. This number was subsequently increased by Li et al. [82] to 2786 but warned that only 2006 could be considered as completely safe and only a few dozen are cultivated commercially.

The most popular are the white button mushroom (*Agaricus bisporus*), paddy straw mushroom (*Volvariella* spp.), oyster mushroom (*Pleurotus* spp.), enokitake mushroom (*Flammulina velutipes*), wood ear mushroom (*Auricularia* spp.) and shiitake (*Lentinula edodes*) [83–85].

Many species are known to produce bioactive metabolites, but the number of species specifically cultivated for their medicinal values is limited to fewer than a dozen species of *Ganoderma*, *Cordyceps militaris*, *Phellinus igniarius* and *Tremella*

Fig. 11.2 Medicinal mushrooms cultivated in China: *Ganoderma leucocontextum* cultivation, Wei-Ping shade house, Tibet Academy of Agriculture and Animal Science (**a**, **b**), Lhasa Konen factory producing *Cordyceps militaris* (**c**, **d**) (Ian Hall pictures)

fuciformis, which are popular especially in Asian countries [17, Ian Hall and Wei Ping personal communication] (Figs. 11.2 and 11.3a).

A majority of the commercially cultivated mushrooms are saprobic feeding on dead or decaying organic matter. Their cultivation can be achieved by inoculating their mycelium in an appropriate substrate and choosing the correct combination of humidity and temperature. The cultivation of edible ectomycorrhizal mushrooms (EEM) is more complicated because most of these fungi need to establish a symbiotic relationship with a suitable host plant to complete their life cycle [87].

Nevertheless, the difficulties and most of the efforts in developing new methods for mushroom cultivation are devoted to EEM for their economic interest. In fact, the most sought-after mushrooms are EEM, like truffles (*Tuber* spp.), porcini (*Boletus edulis* s. l.), chanterelles (*Cantharellus cibarius*) and the milk-cap fungus (*Lactarius deliciosus*) [5, 17, 66, 88].

11.2.1 Nutritional Value of Edible Mushrooms

Edible mushrooms are regarded as an important food resource due to their high-quality protein content [89, 90] and their nutritional properties as excellent sources of vitamins and minerals, for their low-fat content and the large amounts of dietary

Fig. 11.3 Saprobic and ectomycorrhizal cultivated mushrooms: *Ganoderma lucidum* cultivated on agricultural wastes in the greenhouse of Bologna University (Federico Puliga picture) (**a**), *Morchella* cultivation in China (**b**), *Lactarius deliciosus* cultivation in New Zealand (Ian Hall picture) (**c**), *Tuber borchii* orchard in Bologna realized with plants inoculated with pure culture of mycelium (**d**), an ascoma found in the *T. borchii* orchard (**e**), a *Tuber melanosporum* ascoma found under a 3-year oak in South Africa (**f**)

fibre [17, 71, 91]. Although fresh mushrooms contain about 85–95% moisture content and only 3% of protein (from 19% to 37% of dry weight), they have a complete profile of essential amino acids, which can cover the requirements for adults [89].

Mushrooms can be a substitute for animal proteins, and they are particularly important in undeveloped countries, where meat is a limited resource [92]. Moreover, the use of mushroom protein source is a good solution to health and animal welfare concerns that have arisen through traditional meat production and consumption [93]. They are also an ideal food for those committed to a vegan diet and those who need a source for vitamins D and B12, which are scarce or lacking in plant-based diets [94, 95].

Many edible species contain also bioactive compounds, which have medicinal or cosmetic values (**see Parts 3 and 4**).

The button mushroom *(A. bisporus*) and shiitake (*L. edodes*), which are very popular edible mushrooms, possess nutritional proprieties and pharmacological activities [53, 96, 97]. The multiple health benefits of mushrooms are increasing their consumption per capita which has increased 21-fold over the last 56 years [82].

Morels (*Morchella* spp.), which are worldwide appreciated for their unique flavour, have been used in traditional Chinese medicine (TCM) and Western pharmacopeia for centuries, due to their health-related benefits [98]. *Lactarius deliciosus* is another excellent culinary mushroom with good nutritional proprieties which was also shown to have various pharmacological activities, including anticancer, antimicrobial, hypolipidaemic, anti-fatigue, antioxidant and immunomodulatory activities [99].

Although truffles (*Tuber* spp.) have proved to have nutritional and medicinal properties [100], their consumption is generally limited because of their prohibitive prices [17]. The most valuable species, the Italian white truffles (*Tuber magnatum*), the Périgord black truffle (*Tuber melanosporum*), bianchetto truffle (*Tuber borchii*) and Burgundy truffle (*Tuber aestivum*), are generally consumed in a few grams for flavouring other food. *Tuber magnatum* is the most expensive due to its unique taste and flavour, the limited geographical distribution and cultivation difficulties [101]. In the last season (autumn 2021), for example, its retail price ranged between 4000 and 6000 €/kg in Italy. Its prices are more higher in outside growing countries [17] or in dedicated auctions; in the white truffle auction of Alba, for example, an ascoma weighing 830 g was sold at the incredible price of 214.000 €/kg [88], https://www.rte.ie/news/newslens/2021/1115/1260065-white-truffle-italy/ in November 2021. The other truffles command lower prices which vary between 100 and 2000 €/kg depending on the species, the season, the origin and the size of the ascoma.

11.2.2 Progress in Biotechnological Cultivation and Usage

11.2.2.1 Cultivation of Saprobic Edible and Medicinal Mushrooms

The first attempts to cultivate mushrooms date back to the 600 AD when the first wood ear *Auricularia* mushroom cultivation was reported in China. However, until

the advent of modern mushroom cultivation of fungus on the substrates was shear good luck and down to the spontaneous inoculation by spores [102].

Wood was the first substrate used for cultivation of ligninolytic mushrooms like *L. edodes* and *Auricularia* spp. *Agaricus bisporus* was the first mushroom species to be cultivated in compost made of a mixture of substrates like straw, corn cobs, horse and poultry manure, peat moss, gypsum and lime. The origin of biotechnological mushroom cultivation could be associated with the first use of pure cultures in *A. bisporus* by Constantin and Matruchot in 1894 [85]. Further improvement and development of modern technologies and breeding new strains enhanced mushroom productivity particularly over the past 50 years [103].

In 2020, the cultivated mushroom market was estimated at USD$ 16.7 billion (https://www.globenewswire.com/news-release/2020/04/27/2022477/0/en/Global-Mushroom cultivation-Industry-2020-to-2025-Economic-Viability-of-Mushroom-Cultivation-and-Trade-by-Developing-Countries-Presents-Opportunities.html) and is projected to witness significant growth due to the health benefits of mushroom consumption. Moreover, the necessity to find new food resources in order to satisfy the demand of increasing population and of protein from eco-sustainable sources is one of the challenges faced by the cultivation of mushrooms.

The cultivation of saprobic mushroom needs a short time, and the first production begins in a few weeks or maximum several months, depending on the fungal species and the strain, the substrate and climatic conditions [102]. Moreover, mushroom cultivation could utilize different agrowastes as substrates contributing to generating economic development in rural territories. The capability of mushrooms to develop on different wastes makes them the ideal candidates for developing circular economy systems. In this process, the use of the digestate, the material remaining after the anaerobic digestion of a biodegradable feedstock from biogas plants, as substrate for mushroom cultivation is an interesting alternative. Digestate is typically separated mechanically into liquid and solid fractions which are both commonly used as fertilizers [104, 105]. However, there are concerns in their direct use as fertilizer because they are a source of greenhouse gases (N_2O and CH_4), although less than the untreated biomass [106, 107]. Moreover, the solid fraction of digestate from agricultural feedstocks still contain recalcitrant organic compounds prevalent in the lignocellulosic components, which are not degraded during the anaerobic fermentation but may be an excellent carbon source for ligninolytic mushrooms. Fornito et al. [108] showed that *Cyclocybe aegerita*, *P. cornucopiae* and *P. ostreatus* are able to grow and fructify on corn digestate with a biological efficiency (19%, 80% and 103.3%, respectively) similar to those obtained on wood-straw-based traditional substrates. On the other hand, also the liquid fraction of digestate was successfully used in spruce sawdust fermentation for cultivation of *P. ostreatus*, *P. eryngii* and *G. lucidum* [109]. After mushroom cultivation, the spent substrate is useful as fertilizer due to the presence of nutrients and its protective activity against soil-borne diseases. In fact, it has been shown that the changes in microbial composition during mushroom mycelial growth in the substrate and the subsequent increased abundance of beneficial microbes improve its suppressive capacity against several pathogens [110, 111]. Alternatively, the spent substrate after mushroom lignin

degradation can be reused in plants for biogas production or for extraction of bioactive molecules, such as enzymes, or for the extraction of chitin or as feed of animals and in particular invertebrates such as insects or earthworms [108, 112–115]. Worldwide new species and new varieties of mushrooms are being annually added to meet the increasing demand for new mushroom products.

One of the most recent successfully cultivated mushroom is the morel (*Morchella* spp.). In recent years, the outdoor cultivation of true morels has been successful and expanded to a large scale in China after more than 100 years of failures [116] (Fig. 11.3b). The species currently cultivated in China are the saprobic species, the black morels in the Elata clade, in particular *M. importuna*, *M. sextelata* and *M. eximia* [117].

Despite morels have been routinely cultivated in ordinary farmland soils, sometimes there are unsuccessful cases for unknown reasons. These failures could be due to unfavourable soil microbiota [117] or for some genetic and biological aspects which remain poorly understood. Several phases of the life cycle of morels have been not completely unravelled, like the mechanisms of fertilization, including the role of microconidia, the morphogenesis of microsclerotia and factors which trigger fruiting [118, 119].

Moreover, some *Morchella* species in the Esculenta clade establish trophic interactions with the roots of the plants, which at the same time resemble mycorrhizal, saprobic or pathogenic phases; this has not been adequately understood [120, 121]. Fundamental research on morels is obviously necessary to fill the knowledge gaps and for technological progress of *Morchella* for its artificial cultivation [118].

Cordyceps militaris is a medicinal fungus which in nature infects the larvae of lepidopteran host, and over the past decade it has been cultivated in China on artificial media (Fig. 11.2c, d). Several studies were carried out to improve the cultivation medium in order to increase fruiting body formation and extend its bioactive compounds, especially cordycepin [122, 123]. However, the most important of the entomogenous fungi is *Ophiocordyceps sinensis* which is collected in the high grassland areas primarily of Tibet, Nepal and Bhutan where it sells at very low prices. Recently the cultivation of *O. sinensis* fruiting bodies on artificial media on the host caterpillar *Thitarodes* sp. has been successfully established in laboratories of southern China where environmental conditions are mimicked in the wild Tibetan alpine meadows [124].

Ophiocordyceps robertsii is another but lesser known medicinal endoparasitic fungus of insects. Traditionally it is used by New Zealand Maori to produce a dye to colour the moko (body and face carving). A permanent culture was first developed by Wei-Ping Xiong and Ian Hall in 2019 (personal communication) and is now awaiting further study.

Recently, the submerged cultivation of medicinal mushrooms has shown to be a promising and reproducible alternative for the production of mushroom metabolites [125]. In submerged cultivation the mycelium of mushrooms is grown in a liquid medium in which nutrients are dissolved and oxygen supply is reinforced by agitation [126]. It can be achieved in flasks or bioreactor vessels which are more

suitable at industrial levels. Using this technique, physical (temperature, aeration, agitation, etc.) and chemical (pH, medium composition, etc.) factors could be controlled ensuring biomass quality and standardization of metabolite production and opening up the possibility to obtain safely bioactive compounds by the inedible mushrooms [126].

11.2.2.2 Cultivation of Edible Ectomycorrhizal Mushrooms

The edible ectomycorrhizal mushrooms (EEM) live in an intimate association with the roots of suitable trees and shrubs in temperate, boreal and, to a lesser extent, tropical forests [127, 128] providing the host plant with soil nutrients and water and receiving in exchange carbon. The lifestyle of EEM complicates the methods for cultivating these fungi and extends the time of the first production.

Among these species, *T. melanosporum* (the Périgord black truffle) was the first EEM fungus to be successfully cultivated (Fig. 11.3f). Its cultivation was introduced in the early 1800s by the French farmer Joseph Talon. His method was quite simple and consisted of sowing acorns in soils suitable for truffle growth. Truffle cultivation improved considerably over last 70 years when the modern methods of cultivation were introduced. This consisted of inoculating seedlings or cuttings with truffle in greenhouses and then transplanting them in suitable sites. Initially, three different methods of inoculation were proposed: spore inoculum, mother plant technique and mycelial inoculation. Spore inoculum involves inoculating sterile young plants, a few months old, with truffle spores which are obtained by grinding truffles that are fresh, refrigerated or stored in moist sand, dried or frozen [129, 130]. The mother plant technique involves planting seedlings into the rooting zone of a plant known to be mycorrhized with the required truffle and mycelial inoculation using pure culture of *Tuber* mycelium. The mother plant technique was soon abandoned because of the high risk to spread contaminant ectomycorrhizal fungal species accidentally present on the mother plant. The mycelial inoculation was used only for experimental purposes and to overcome the difficulties in obtaining pure cultures of *Tuber* mycelium [130]. Thus, spore inoculum has become the method used by all the companies producing *Tuber* plants because it is simple and effective for most of the species of valuable *Tuber* spp. However, due to the high cost of *Tuber* ascomata, batches of truffles, which contain small, broken and often completely rotten ascomata, are often used as inoculum. That makes it very difficult for identification of any ascoma, and those of less valuable *Tuber* species can escape the control and are included in the inoculum. That increase the risk of contamination of the root of the plants with undesired mycorrhizal species, and, for example, plants which should be mycorrhized with *T. melanosporum* are instead infected with the similar but less valuable *Tuber brumale* or with other worthless *Tuber* spp. Fortunately, in the last 30 years, morphological and molecular methods to identify ascomata and mycorrhizas have been perfected [86, 130, 131]. In France and in some regions of Italy, both the ascomata used as inoculum and the mycorrhizas are routinely checked to avoid the production and commercialization of plants carrying mycorrhizas different from those declared by the nursery [132–134].

Since the truffle spores are derived by meiosis of a virtual zygote, they are genetically different and of unknown genotypes [135]. That could be a potential adaptive advantage when the soil and climatic condition of the plantation site are unknown but do not allow a genetic selection of the best fungal genotypes for each specific ecological condition and the possibility to improve the productive performances of truffle orchards. The recent positive results obtained in inoculating plants with mycelial pure culture and the first production obtained by planting seedlings inoculated with different mycelial strains of *T. borchii* open up the possibility of commercially applying this method [136] (Fig. 11.3d and e). This will allow selecting the strains producing ascoma of best aroma composition or more adaptable to climatic conditions, characters that seem to be genetically controlled [137, 138].

Mycelial inoculation is also applied to produce *Lactarius deliciosus* mycorrhizal plants. Its cultivation was introduced in New Zealand in the late 1990s; it spread later to Europe and was introduced into China around 2014. The fruiting body production has been estimated to be as high as 1–3 tonnes per hectare in New Zealand [139] (Fig. 11.3c).

11.3 Mushroom-Derived Bioactive Molecules

11.3.1 Polysaccharides

The polysaccharides (β-1,3 and β-1,6 glucans) are one of the major bioactive molecules in agaricomycete and ascomycetes mushrooms with significant immunomodulatory, antioxidant, antimicrobial and other medicinal effects. Fungal polysaccharides (β-glucans) lentinan, krestin, schizophyllan and pleuran with commercial application were extracted from *L. edodes*, *P. ostreatus*, *Trametes versicolor* and *Schizophyllum commune* [5, 8, 140–150].

The β-glucans and their bioactivity were also reported from other mushrooms, such as *A. bisporus*, *Auricularia auricula-judae*, *Ganoderma* spp. and *Suillus granulatus* [151, 152]. *Ganoderma* polysaccharides have particularly been suggested as a healthy dietary food for cancer patients [153].

11.3.2 Terpenoids and Phenolics

Inedible and edible medicinal mushrooms may be sources of phenolic compounds and derivatives. Fungal phenolics possess anti-carcinogenic, anti-inflammatory, antioxidant and anti-mutagenic effects [8, 26, 154–156].

Recent studies showed that *Agaricus campestris*, *A. bisporus*, *B. edulis*, *C. cibarius*, *Grifola frondosa*, *Macrolepiota procera*, *P. ostreatus*, *Russula alutacea*, *R. vesca*, *S. commune*, *T. versicolor*, *Trametes gibbosa* and *Volvariella volvacea* were considered as source of bioactive phenolics (flavonoids, β-carotene, lycopene, coumarins, phenolic acids) with different therapeutic effects [156–

164]. Among these, species from order Boletales (*Boletopsis leucomelas*, *Boletus grisea*, *Paxillus curtisii* and *P. panuoides)* are especially rich in pigments of various phenolic origins for potential medicinal exploitation [165].

A variety of bioactive terpenoids represents another unexploited group of lipid derivatives in mushrooms. The chemical structures of several fungal terpenoids have been determined [166, 167].

Edible and medicinal mushrooms, such as *Ganoderma* spp., *Pleurotus* spp., *Fomitopsis palustris*, *Fomitopsis betulina* and *Tricholoma pardinum*, contain lanostane triterpenoids (pardinols A–H and saponaceol B) with antibacterial, antimitotic, antiviral, cytotoxic, immunomodulatory and other therapeutic effects [8, 46, 147, 168–174].

The sesquiterpenoid eremophilanes with antibacterial, anti-inflammatory, antiobesity, antiviral and cytotoxic effects were detected in *Xylaria* mushrooms [175], as well as in submerged cultures of *Inonotus* sp. [176]. The ergostane and lanostane triterpenoids were identified in *Antrodia cinnamomea* [177]. Cytotoxic sesquiterpenes derived from the fruiting bodies of *Russula* spp. [166], lanostane triterpenoids from *Piptoporus betulinus* [178] and hypoglycaemic triterpenes from medicinal mushroom *Wolfiporia cocos* [179] have also been reported. Bioactive meroterpenoid suillin and related pigments with antimicrobial, anti-mitogenic, antioxidant and apoptosis-inducing effects against human cancer cell lines were detected in *Suillus placidus* [180] and *Suillus bovines* [181]. Suillin was suggested as an effective agent to treat liver cancer. A new lipid peroxidation inhibitor bolegrevilol was detected in the edible mushroom *Suillus grevillei* [182].

11.3.3 Lipids and Sterols

Evaluation of lipid and sterol content (ergosterol, fungisterol, lanosterol, cholesterol, cerevisterol and derivatives) of mushrooms from *Amanita*, *Boletus*, *Lactarius*, *Suillus*, *Tricholoma*, *Tuber* and other genera showed that they differ in total lipid quantities and fatty acid composition [5, 8, 17, 163, 183]. Among 20 different fatty acids present in mushrooms, the more common are oleic, linoleic and palmitic acids followed by stearic acid. Mono- and polyunsaturated fatty acids, including oleic and linoleic, are considered as valuable food supplements for human diet and nutrition [6].

Steroids, lanostane and ceramide derivatives were originally isolated and identified from the methanolic extract of agaricomycete species *Scleroderma bovista* [184]. Among these, the lanostane derivatives showed significant anti-proliferative properties against human cancer cell lines HeLa, A2780, MDA-MB-231 and MCF-7.

11.3.4 Lectins

Mushrooms possess bioactive proteins, such as lectins, ribosome-inactivating proteins and fungal immunomodulatory proteins (FIPs).

Lectins are non-enzymatic proteins that specifically interact with sugars. They possess immunomodulatory, mitogenic, cytotoxic, antitumor and antimicrobial activities making them as potential therapeutic agents. Different lectins were isolated from fruiting bodies and mycelia of Agaricomycetes genera *Amanita*, *Boletus*, *Laccaria*, *Lactarius*, *Russula* and *Tricholoma* [185, 186]. Lectins with immunomodulating and hypotensive effects were isolated from *Tricholoma mognolicum* [187].

The potent antitumor and anti-proliferative lectins (homodimeric, 60 kDa) towards human hepatoma HepG2 and human breast cancer MCF-7 cells were isolated from *Russula lepida* and *R. delica* [188, 189]. These lectins against murine leukemic L1210 cells from *Lactarius flavidulus* [190] and haemolytic toxic lectin against murine and human leukemic cell lines were obtained from *Amanita virosa* [191]. Antiviral and anti-proliferative lectin derived from *B. edulis* inhibited human viral reverse transcriptase, and the proliferation of several malignant cell lines, by binding the neoplastic cell-specific T-antigen disaccharide Gal β1-3GalNAc, has been reported [189, 192]. A lectin from *Xerocomus (= Boletus) spadiceus* induced a mitogenic response in murine splenocytes [193], while ingestion of lectins from *Boletus venenatus* showed fatal toxicity in mice [194]. Lectin TBF-1 specific to the hypogeal ascomata was identified in *T. borchii* [70].

The galectins are a class of bioactive proteins that bind specifically to β-galactoside sugars and have been described in Agaricomycetes *Coprinopsis cinerea* and *Laccaria amethystina* [195, 196].

11.4 Medicinal Properties of Mushrooms

Mushrooms as medicines were recognized nearly 2000 years ago. They are rich source of pharmaceutical constituents for different exploration potential. Traditional medicine and scientific research data showed that both edible and inedible mushrooms possess promising pharmacological potential (antimicrobial, anti-inflammatory, antioxidant, antiviral, cardio-, hepato- and neuroprotective, cytotoxic, hypotensive, immunomodulatory, etc.) and may be considered sources of myco-pharmaceuticals, nutriceuticals or dietary supplements and cosmeceuticals [1, 5, 8, 9, 15, 17, 19, 28, 31–33, 140].

Edible medicinal agaricoid *Pleurotus* mushrooms are mainly known due to their hypoglycaemic, hypocholesterolaemic, neurotoxic and cardioprotective properties [5, 8, 9, 17, 50, 53–57, 59, 60].

The pharmacological potential of inedible bracket fungi, such as *Ganoderma* spp., *T. versicolor*, *Phellinus linteus*, *G. frondosa*, *Fomes fomentarius* and *Fomitopsis pinicola*, could be used to develop health-enhancing functional food products [8, 13, 197–200]. The extracts from mycelia and ascomata of the most

priced medicinal ascomycete mushroom *Ophiocordyceps sinensis* and *C. militaris* possess immunomodulatory and cell apoptosis-inducing activities [201, 202].

Edible ascomycete mushrooms, such as morels and truffles, besides their excellent culinary values, have medicinal properties due to bioactive compounds, dietary fibres, vitamins, polysaccharides, proteins and trace elements. The fruiting bodies and mycelium of *M. esculenta* possess antioxidant activity because of linoleic acid and beta-carotene contents [67]. Polysaccharides isolated from *M. esculenta* showed anti-inflammatory, antitumor, antimicrobial and wound-healing properties [68, 203]. Many truffles and truffle-like fungi (*Picoa* spp., *Terfezia boudieri*, *T. claveryi*, *Tirmania nivea*, *T. pinoyi*, *T. melanosporum*, *Tuber indicum*, *T. sinense*, *T. aestivum* and *T. himalayense*) possess antioxidant, antimicrobial, anti-mutagenic, antitumor and neuroprotective properties [5, 72–75].

11.4.1 Antimicrobial and Antiviral Activity

The prevention and treatment of bacterial, fungal and viral diseases remain a serious problem in modern medicine. Agaricomycete and ascomycete mushrooms are known as active producers of antimicrobial and antiviral compounds: velutin and flammulin from *F. velutipes*; ganodermadiol, ganomycin and ganoderiol from *G. lucidum*; lentinan from *L. edodes*; schizophyllan from *S. commune*; krestin from *T. versicolor*; and others [8, 204–211].

The antimicrobial activities of extracts from *A. bisporus* and *T. gibbosa*, against Gram-positive and Gram-negative bacteria, as well as phytopathogenic and keratinophilic fungi, have been reported [212]. The bacteriostatic and bacteriocidical effects against *Helicobacter pylori* bacteria were revealed using ethanolic extracts of *A. bisporus*, *Coprinus comatus*, *C. militaris*, *F. velutipes*, *G. lucidum*, *G. frondosa*, *Hericium erinaceus*, *Hypsizygus marmoreus*, *Ganoderma applanatum*, *L. edodes*, *Ph. igniarius*, *P. eryngii* and *P. ostreatus* [37, 213, 214].

The aqueous extracts of edible ascomycete mushrooms *Picoa juniperi*, *T. claveryi* and *T. pinoyi* (desert truffles) showed in vitro antibacterial activities against Gram-positive human pathogenic reference strain *Staphylococcus aureus* ATCC 29213 and Gram-negative *Pseudomonas aeruginosa* strain ATCC 15442. The acid-soluble protein extracts of *T. pinoyi* and *T. claveryi* have minimum inhibitory concentrations (MIC) of 50 μg/mL against tested pathogens [215].

The antiviral activities of edible agaricoid mushrooms *B. edulis*, *L. edodes*, *P. ostreatus* and *Lignosus rhinocerotis* against herpes simplex virus (HSV) type 1, human papillomavirus (HPV) and dengue virus type-2 (DENV-2) were reported [209, 216, 217]. Extracts from mycelia of polyporoid species *Daedaleopsis confragosa*, *Datronia mollis*, *Ischnoderma benzoinum*, *Laricifomes officinalis*, *Lenzites betulina*, *T. gibbosa* and *T. versicolor* showed antiviral activity against influenza A virus (H_5N_1 and H_3N_2) [218]. It has been revealed that polysaccharides, glycoproteins, melanins, nucleosides, proteins and terpenoids from several Agaricomycetes exhibit antiviral effects against hepatitis, herpes, human immunodeficiency virus (HIV), influenza, West Nile viruses as well as orthopox viruses,

including the variola virus [211]. Tested mushrooms were suggested as perspective agents to develop novel antiviral myco-pharmaceuticals.

Mycelia of several polyporoid *(Auriporia aurea*, *F. fomentarius* and *T. versicolor)* and agaricoid (*P. ostreatus*, *P. eryngii*, *F. velutipes* and *Lyophyllum shimeji)* mushrooms inhibited the reproduction of influenza A (H_1N_1) and herpes simplex (HSV-2) viruses [207]. Among tested samples *T. versicolor* 353 strain was detected as a source of low toxicity antiviral agent. The antiviral activity was reported in ascomycete *Morchella conica*, *M. esculenta* and *T. boudieri* [205]. However, antiviral mushroom-derived bioactive molecules and mechanisms of their action remain subjects for further research.

Several bioactive compounds of mushrooms (polysaccharides, proteins, terpenes, melanins, etc.) are exhibiting an antiviral activity with combination of immunomodulatory, immunosuppressive and anti-inflammatory properties which may be safely used in the prevention and treatment of respiratory viral infections [211, 219–221].

The coronavirus disease 2019 (COVID-19), a de novo pattern of pneumonia, has caused pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). COVID-19 is associated with several comorbidities; therefore, different preventive and curing therapies should be applied. Medicinal mushrooms may be a good candidate for preventive and therapeutic use against COVID-19 [61, 222]. The combination of immune cell activation with a moderate impact on inflammatory cytokines may be beneficial in patients with COVID-19 [223].

Rahman and coauthors [61] described Reishi or Lingzhi (*G. lucidum*) as the most suitable anti-COVID agent. Another potential candidate against the SARS-CoV-2 virus may be chaga mushroom (*I. obliquus*) commonly grown in Asia, Europe and North America. This bracket fungus is characterized by a wide range of antiviral compounds and it is used as a raw material in various therapies. Antiviral melanin from wild and cultivated *I. obliquus* strain is effective against the pandemic strain of influenza virus A/California/07/09 (H1N1 pdm09) [221, 224]. Other medicinal mushrooms are also promising; for example, cell-based studies show a reduction in the production of proinflammatory cytokines by β-glucan-rich extracts obtained from *L. edodes* in COVID-19 patients. Fruiting bodies and mycelia of agaricomycete mushrooms *Agaricus blazei* (AbM), *H. erinaceus* and *G. frondosa* have been reported as antiviral, antibacterial, immunomodulatory and anti-inflammatory agents and may be suited for treatment of pneumonia caused by COVID-19 infection. A mushroom extract-based biotech product Andosan™ containing extracts from these fungi has shown significant antibacterial and anti-inflammatory effects and increased survival in mice with pneumococcal sepsis. Therefore it can be suggested as a prophylactic or therapeutic agent against severe pneumonia that often complicates COVID-19 infection [222]. However, data with chemically well-defined fungal preparations to support COVID-19 patients are necessary to further evaluate for their medicinal properties at certain stages of the disease [223].

11.4.2 Immunomodulatory and Antitumor

The immunomodulatory activity is the most prominent medicinal property of mushrooms [8, 33, 47, 140, 153, 225–228]. β-Glucans schizophyllan, lentinan, krestin and grifolan, as well as proteins, glycoproteins and lipopolysaccharide (LPS), have been identified as immunomodulators. They are widely used in the treatment of several types of cancer [8, 33, 206]. Mushroom glucans prevent oncogenesis and exhibit antitumor effects by inducing immune response in the host [142, 144, 229–232]. They may also modify cell cycle-regulating genes and induce apoptosis [233].

Glucan grifolan or maitake D-fraction isolated from *G. frondosa* stimulated the production of granulocyte colony-stimulating factor (CSF) and recovery of peripheral blood leukocytes [234] and showed anti-metastatic effect [233, 235]. The immunomodulatory, cytotoxic and apoptosis-inducing effects of β-glucans from *Ganoderma* spp., *Inonotus obliquus* (Chaga mushroom) and *W. cocos* are supported by the presence of triterpenoids [227, 236]. The immunomodulatory polysaccharides isolated from *Macrocybe titans* and *Collybia radicata* were suggested for further application as food and pharmaceutical agents [228, 237]. A ganoderic acid and FIPs with anti-proliferative and apoptosis-inducing activities have been reported in different *Ganoderma* mushrooms [47, 238, 239].

Immunomodulatory, anticancer and anti-inflammatory effects were revealed in medicinal mushrooms *B. edulis* [240], *Coriolus* (syn. *Trametes*) *versicolor* [241], *Inonotus hispidus* [242], *L. edodes* [243], *P. ostreatus* [244] and *Taiwanofungus camphoratus* [245]. Further myco-pharmacological studies will promote the transfer of mushroom-derived biomolecules to clinically effective therapeutics [8, 140, 246].

11.4.3 Antioxidant and Anti-inflammatory

Edible and medicinal mushrooms are recognized as a natural source of phenolics, polysaccharides and terpenoids with antioxidant and anti-inflammatory effects [8, 19, 21, 43, 62, 153, 247–260].

The antioxidant activity due to high content in total phenols and flavonoids was revealed in methanolic extracts of 29 wild edible mushrooms. The extracts of *Cantharellus cibarius*, *C. cinereus*, *Craterellus cornucopioides* and *Hydnum repandum* exhibited high cytotoxicity and induced apoptosis-necrosis in A549 cells. As an active ingredient, anti-proliferative piceatannol was originally reported in tested species [261].

Fermentation broth samples of three *Ganoderma* spp. contain a large number of phenolic derivatives and flavonoids with antioxidant and antimicrobial activities and were suggested as source of antioxidant and antimicrobial agents [141, 251]. It was shown that the treatment with extract of *A. brasiliensis* improved the antioxidant defences, diminished by rheumatoid arthritis [252].

A significant antioxidant effect was revealed in methanolic extracts from mycelia of *Pleurotus* spp. The usage of mycelial extracts as dietary supplement can prevent the process of oxidative damage [50, 253].

Antioxidant and anti-proliferative effects were revealed in Boletales *(B. edulis)* and other edible and medicinal agaricoid mushrooms (*A. subrufescens*, *A. auricular-judae*, *F. velutipes*, *Ganoderma capense*, *H. erinaceus*, *L. edodes*, *Pleurotus djamor*, *S. luteus* and *W. cocos)* [40, 49, 249, 256, 258, 262–264].

Antioxidant and anti-aging activities of polysaccharides isolated from ascomycete fungus *Cordyceps cicadae* have recently been observed [265]. Anti-inflammatory effect of polysaccharides, as well as aqueous, ethanolic and methanolic extracts from *Lactarius rufus* [266], *L. edodes* [267], *P. giganteus* [51] and *L. rhinocerotis* [268], was revealed.

Further studies are needed to elucidate the antioxidant and anti-inflammatory potential of mushrooms and their usage as healthy food biotech products due to the synergistic effects of all the bioactive molecules present (polyphenols, polysaccharides, vitamins, carotenoids and minerals) [144, 155].

11.4.4 Anti-metabolic Syndrome

The metabolic syndrome (MS) is a pathological condition including hyperglycaemia, hyperlipidaemia, insulin resistance, obesity and hypertension. These symptoms are important signs of type 2 diabetes and increase the risk of cardiovascular diseases (CVD) [9, 18, 22, 269–271].

Currently, the existing drugs such as insulin, statins and angiotensin-converting enzyme (ACE) inhibitors used for the treatment of MS have limited therapeutic efficacy and several side effects. There are other drugs (e.g., HMG-CoA reductase, aldose reductase and α-glucosidase) which are also used in MS therapy. Nevertheless, considerable effort has been made to develop new preparations and pharmaceuticals to ameliorate the glucose and lipid metabolism without significant side effects.

Mushroom-rich nutrition is regarded as dietary healthy food to prevent and cure MS pathology. Recent studies revealed huge anti-MS potential in *A. bisporus*, *A. brasiliensis*, *G. lucidum*, *G. frondosa*, *H. erinaceous*, *Ph. linteus* and *Pleurotus* spp. [3, 8, 19, 38, 272–276]. Moreover, the eritadenine extracted from *L. edodes* has been identified as an anti-atherogenic agent with ACE-inhibitory activity [277]. New bioactive compounds including lanostane triterpenoids isolated from *G. lucidum* were suggested to control hyperglycaemia and hyperlipidaemia, as well as to cure the MS [35].

The hypolipidaemic and hypoglycaemic properties of several agaricoid and polyporoid species, such as *Calocybe indica*, *P. ostreatus*, *P. giganteus*, *V. volvacea* and *Inocutis levis*, have been reported [271, 278–280]. However, data concerning the molecular mechanisms of their therapeutic action are still not sufficient [281].

Hypolipidaemic and hypoglycaemic properties of agaricomycete and ascomycete mushrooms allow using them as a healthy food to prevent CVD [9]. Among these species *G. frondosa*, *L. edodes* and *P. ostreatus* are almost ideal for low-calorie diets due to a high content of fibre, proteins and microelements [282, 283].

Recent studies have demonstrated that bioactive molecules (i.e., terpenoids, peptides, isoflavones as biochanin A and formononetin, lanosterone derivative as fomiroid A and lovastatin) derived from *Boletus aestivalis*, *Clitocybe nuda*, *G. lucidum*, *G. frondosa*, *H. marmoreus*, *L. edodes* and *Pleurotus* spp. can regulate the levels of low, high-density lipoproteins, total cholesterol and fasting triglycerides and prevent the development of arterial hypertension, oxidative stress, diabetes and CVD [8, 9, 284].

11.4.5 Neuroprotective

The age-related neurodegenerative diseases (NDD) are affecting millions of people worldwide. Oxidative stress, mitochondrial dysfunction, inflammation and axonal transport deficits play a significant role in the development of NDD. The general strategies to prevent the progression of NDD are physical activity, stress-free lifestyle and healthy diet, enriched with different natural supplements. Therefore, it is urgent to explore natural neuroprotective agents, including myco-pharmaceuticals and myco-food to prevent and mitigate development and symptoms of age-related NDD [15, 16, 285, 286].

Hericium species [*H. coralloides*, *H. erinaceus*, *H. flagellum* (syn. *H. alpestre*)] are among the highly praised edible mushrooms, as producers of neuroprotective biomolecules, such as hericerin, hericenones, erinacines and corallocins [16, 274, 287–290].

Several medicinal mushrooms, such as *Antrodia camphorate* and *G. lucidum*, possess neurotrophic effects due to chemical contents of bioactive compounds (alkaloids, fatty acids, lectins, lipids, polysaccharides, phenolics, polyketides, terpenoids, sterols, etc.). They are considered natural agents in the management of different neurodegenerative disorders, including depression, Alzheimer's, Huntington's and Parkinson's diseases [16, 291–296].

The role of edible and medicinal agaricomycete and ascomycete mushrooms (*A. bisporus*, *A. brasiliensis*, *C. militaris*, *G. lucidum*, *G. frondosa*, *H. erinaceus*, *L. edod*es, *Lignosus rhinocerus*, *O. sinensis*, *P. giganteus*, *T. versicolor*, *Termitomyces albuminosus* and *T. fuciformis*) in the treatment of NDD and the study of molecular mechanisms of neuroprotective and cognitive effects have recently been reported [16, 296–299].

Further efforts are warranted to discover the neuroprotective mechanism of mushroom-derived biomolecules [8, 16, 285, 286, 300].

11.5 Advances in Production of Mushroom-Derived Biotech Products

A wide spectrum of bioactivities of mushroom-derived compounds could be used to develop health-enhancing biotech products for human and animal use [17, 301, 302]. Mushroom pharmaceuticals, nutriceuticals, nutraceuticals and cosmeceuticals possess different therapeutic effects, such as anticancer, antioxidant, anti-inflammatory, immunomodulatory, cardioprotective, neuroprotective, etc. [8–10, 14–16, 274, 287, 294, 295, 303–306].

Several edible and medicinal mushrooms (*A. subrufescens*, *Ganoderma* spp., *G. frondosa*, *H. erinaceus*, *L. edodes*, *Laetiporus sulphureus*, *Ph. linteus*, *P. ostreatus* and others) are considered a rich source of innovative biomedical compounds to develop myco-pharmaceuticals. They can be extracted not only from fruiting bodies but also from mycelial biomass and cultural broth [5, 8, 14, 17, 62, 301, 307, 308].

Nutraceutical ("nutrition" and "pharmaceuticals") is any substance which may be considered a food or part of the food and provides some medical or health-enhancing effects, including prevention and curing of the diseases. Agaricomycete and ascomycete mushrooms (*A. bisporus*, *Auricularia* spp., *Pleurotus* spp., *B. edulis*, *F. velutipes*, *L. edodes*, *V. volvacea*, *M. esculenta*, *T. borchii*, *T. melanosporum,* etc.) due to their volatile compounds are regarded not only as gourmet food but also nutraceuticals with high nutritional and dietary values for human wellness [5, 17, 283, 295, 310, 312, 321, 328, 330, 338, 341, 343, 344].

The nutraceutical and pharmaceutical potential of mushroom bioactive molecules derived from *A. bisporus* (lectins), *A. auricula-judae* (acidic polysaccharides), *G. frondosa* (grifolan, lectin), *Lentinus* (= *Pleurotus*) *sajor-caju* (lovastatin) and *O. sinensis* (cordycepin) has been evaluated. Several nutraceutical and pharmaceutical biotech products derived from these fungi were approved for clinical use in many countries [316, 326, 332, 339, 344].

The mushroom nutraceuticals and dietary supplements can be obtained from fruiting bodies, mycelia, sclerotia and spore powder. The supplementation of different types of health food products (dairy beverages, yogurts, bread, pasta, beer) with mushrooms increases their quality and nutritional values [17, 71, 80, 315, 317, 319, 320, 322, 324, 333, 344, 346, 347, 353].

Several white-rot agaricoid, polyporoid, hymenochaetoid and russoloid Agaricomycetes fungi are used in production of beverages, wine, beer, cosmeceuticals, prebiotics, functional foods and nutraceuticals, for stabilisation and delignification of feedstock, as well as in baking [340].

It was showed that polysaccharides isolated from cultivated ascomycete fungus *O. sinensis* modulate intestinal mucosal immunity and gut microbiota in cyclophosphamide-treated mice [352]. Agaricomycetes species *C. versicolor*, *G. lucidum*, *G. frondosa*, *H. erinaceus*, *I. obliquus* and *L. edodes* have also been reported as prebiotics due to fungal glucans regulating gut microbiota in the host [226, 327, 336, 345].

The vitamin-enriched mushroom dietary food could play an important role in the prevention of chronic diseases [334].

Currently, mycelial cultivation industry is progressing, and the production of mycelium-derived mushroom biotech products is constantly improving [125, 142, 143, 313]. Recent progress in fungal biology and biotechnology, genomics, proteomics and myco-pharmacology has contributed to usage of agaricomycete and ascomycete mushrooms in medicine and food industries [335].

Cosmeceuticals are the products between cosmetics and pharmaceuticals containing bioingredients with anti-aging, anti-inflammatory, antioxidant and anti-pigmentative effects.

Currently, the cosmetic industry is in a constant search for anti-aging (anti-collagenase, anti-elastase, anti-hyaluronidase, anti-inflammatory, antioxidant and anti-tyrosinase) biomolecules or extracts. Edible and medicinal mushrooms as unlimited source of bioactive compounds (phenolics, glucans and other polysaccharides, terpenoids) may be considered as valuable sources of cosmetic bioingredients used in formulation of skin and hair care organic cosmeceuticals, nutriceuticals and nutraceuticals [1, 10, 17, 30, 259, 311, 348, 351].

Wild or cultivable mushrooms, such as *A. subrufescens*, *A. bisporus*, *A. auricula-judae*, *O. sinensis*, *Ganoderma lingzhi*, *G. lucidum*, *G. frondosa*, *Hypsizygus ulmarius*, *I. obliquus*, *L. edodes*, *Polyporus* and *Phellinus* species, *S. commune*, *T. versicolor*, *T. fuciformis* and *Tuber* spp., are also incorporated in the formulation of many cosmetic products [1, 10, 11, 17, 29, 30, 325, 342, 351].

Numerous mushroom-derived cosmeceuticals (applied topically, i.e. creams, lotions and ointments) and nutricosmetics (administered *per os*) with different formulations are available in the market. Their usage is significantly high due to minimal regulation and safety compared to traditional drugs. The cosmetic brands used in mushroom ingredients are Bliss (Hut.com Ltd, Cheshire, UK), La Roche (F. Hoffmann-La Roche Ltd, Basel, Switzerland), Nu-Derm (Obagi Medical Products Inc., Irvine, CA, USA), SensiClear (Mission Scientific Skincare Inc., Gold River, CA, USA) and others [10, 17, 351].

11.5.1 Medicinal Mushrooms in Animal Alimentation

Recently, mushrooms have been receiving a great attention as supplement in animal alimentation [314]. Edible and medicinal mushrooms are used as organic food additives for pets for preventing cancer and supporting their optimal immune health [34], https://thenaturalpetdoctor.com/mushrooms-to-naturally-improve-pet-health/). Mushrooms, particularly *Ganoderma* and *Pleurotus* species, can be successfully used in poultry diets for improving the performance of broilers [323, 329]. They have different beneficial effects such as immunomodulatory, antibacterial, antiviral and anti-parasitic and can be used as growth promoters or as an alternative to antibiotics [329].

Infectious bursal disease (IBD), also called Gumboro disease, is one of the most widespread immunosuppressive avian diseases, caused by a highly contagious virus.

IBD vaccination has been used in the chicken industry worldwide to prevent IBD infection. However, IBD vaccines do not completely protect chicken against infectious diseases due to immunosuppressive effects [349]. Ogbe and collaborators [337] showed that the inclusion of about 0.2% of *G. lucidum* fruiting bodies to the feed enhances immune response of chicken vaccinated against IBD.

Coccidiosis is a crucial parasitic disease of the poultry industry which causes enormous global economic losses. Due to the increased resistance to the conventional anti-coccidiosis agents, there is a continuous need to find new anti-coccidials [318]. The anti-coccidial activity of aqueous extract of *G. applanatum* on broiler chicken was recently proved [309].

Least and not last, mushroom dietary supplementation can also improve the poultry meat quality. The inclusion of 10 or 20 g of *P. ostreatus*/kg in the diet of Japanese quails was effective in delaying the lipid oxidation of breasts and enhancing the colour, pH, water holding capacity, cooking loss weight and texture which are parameters to define meat quality [350].

An indirect use of mushrooms for animal alimentation is to use the spent mushroom substrate as feed sources for insects, as, for example, *Tenebrio molitor* larvae, which in turn can be used as feed for poultry or fish [331].

11.6 Conclusions and Future Prospectives

Agaricomycete and ascomycete mushrooms are a source of multifunctional bioactive compounds with broad spectrum of pharmacological activities which can be used to develop commercial biotech products, such as pharmaceuticals, nutriceuticals, nutraceuticals and cosmeceuticals. Edible mushrooms have a great impact on agriculture, environment and economic development in the society.

Advances in fungal biology and biotechnology, edible and medicinal mushroom cultivation industry, as well as myco-pharmacology are addressing to further exploitation of mushroom resources to improve human welfare and promote economic growth. The increased usage of mushrooms and mushroom-derived products can be expected. In exploitation of mushroom resources, it is important to direct the efforts toward their biotechnological cultivation for production of fruiting bodies, mycelia or spores to develop and formulate innovative and standardised mushroom products (nutraceuticals, dietary supplements or cosmeceuticals) and to establish suitable parameters for their quality control.

Further clinical and pharmacokinetic studies of mushroom-derived products and comprehensive assessment of their nutritional values will expand our knowledge for sustainable manufacturing of high-quality standardized biotech products.

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References

1. Alkan S, Uysal A, Kasik G, Vlaisavljevic S, Berežni S et al (2020) Chemical characterization, antioxidant, enzyme inhibition and antimutagenic properties of eight mushroom species: a comparative study. J Fungi 6:166
2. Azeem U, Hakeem KR, Ali M (eds) (2020) Fungi for Human Health. Current Knowledge and Further Perspectives. Springer Nature, Switzerland, Cham, p 113
3. Badalian SM, Serrano JJ (1999) Hypoglycemic activity of the medicinal mushroom *Hypholoma fasciculare* (Fr.) Kumm. Int J Med Mushrooms 1:245–250
4. Badalyan SM (2003) Antioxidant activity of culinary-medicinal mushroom *Flammulina velutipes* (Curt.:Fr.) P. Karst. (Agaricomycetideae). Int J Med Mushrooms 5:277–286
5. Badalyan SM (2012) Medicinal aspects of edible ectomycorrhizal mushrooms. In: Zambonelli A, Bonito G (eds) Edible ectomycorrhizal mushrooms, current knowledge and future prospects, vol 34. Springer-Verlag, Berlin/Heidelberg, pp 317–334
6. Badalyan SM (2016) Fatty acid composition of different collections of coprinoid mushrooms (Agaricomycetes) and their nutritional and medicinal values. Int J Med Mushrooms 18:883–893
7. Badalyan SM (2020) Medicinal coprinoid mushrooms (Agaricomycetes) distributed in Armenia (Review). Int J Med Mushrooms 22:257–267
8. Badalyan SM, Barkhudaryan A, Rapior S (2019) Recent progress in research on the pharmacological potential of mushrooms and prospects for their clinical application. In: Agrawal DC, Dhanasekaran M (eds) Medicinal mushrooms: recent progress in research and development. Springer Nature, Singapore, pp 1–70
9. Badalyan SM, Barkhudaryan A, Rapior S (2021) The Cardioprotective Properties of Agaricomycetes Mushrooms Growing in the Territory of Armenia (Review). Int J Med Mushrooms 23:21–31
10. Badalyan SM, Barkhudaryan A, Rapior S (2022) Macrofungi as Cosmeceuticals: a review. Int J Med Mushrooms 24(3) (in press)
11. Badalyan SM, Borhani A (2019) Medicinal, nutritional and cosmetic values of macrofungi distributed in Mazandaran province of northern Iran (review). Int J Med Mushroom 21:1099–1106
12. Badalyan SM, Gharibyan NG (2016) Diversity of polypore bracket mushrooms, Polyporales (Agaricomycetes) recorded in Armenia and their medicinal properties. Int J Med Mushrooms 18:347–354
13. Badalyan SM, Gharibyan NG (2020) Pharmacological properties and resource value of hymenochaetoid fungi (Agaricomycetes) distributed in Armenia: Review. Int J Med Mushrooms 22:1135–1146
14. Badalyan SM, Rapior S (2020) Perspectives of biomedical application of macrofungi. Curr Trends Biomed Eng Biosci 19:ID556024
15. Badalyan SM, Rapior S (2021) Agaricomycetes mushrooms (Basidiomycota) as potential neuroprotectants. Ital J Mycol 50:30–43

16. Badalyan SM, Rapior S (2021) The Neuroprotective Potential of Macrofungi. In: Agrawal D, Dhanasekaran M (eds) Medicinal Herbs and Fungi – Neurotoxicity vs. Neuroprotection. Springer Nature, Singapore, pp 37–77
17. Badalyan SM, Zambonelli A (2019) Biotechnological exploitation of macrofungi for the production of food, pharmaceuticals and cosmeceuticals. In: Sridhar KR, Deshmukh SK (eds) Advances in Macrofungi: diversity, ecology and biotechnology. CRC Press, Boca Raton, pp 199–230
18. De Silva DD, Rapior S, Hyde KD, Bahkali AH (2012) Medicinal mushrooms in prevention and control of diabetes mellitus. Fungal Divers 56:1–29
19. De Silva DD, Rapior S, Sudarman E, Stadler M, Xu J et al (2013) Bioactive metabolites from macrofungi: ethnopharmacology, biological activities and chemistry. Fungal Divers 62:1–40
20. De Silva DD, Rapior S, Fons F, Bahkali AH, Hyde KD (2012) Medicinal mushrooms in supportive cancer therapies: an approach to anti-cancer effects and putative mechanisms of action. Fungal Divers 55:1–35
21. Du B, Zhu F, Xu B (2018) An insight into the anti-inflammatory properties of edible and medicinal mushrooms. J Funct Foods 47:334–342
22. Friedman M (2016) Mushroom polysaccharides: chemistry and antiobesity, antidiabetes, anticancer, and antibiotic properties in cells, rodents, and humans. Foods 5:80
23. Grienke U, Zöll M, Peintner U, Rollinger JM (2014) European medicinal polypores – a modern view on traditional uses. J Ethnopharmacol 154:564–583
24. Hawksworth DL (2001) Mushrooms: the extent of the unexplored potential. Int J Med Mushrooms 3:333–337
25. Hilszczańska D (2021) Healing Properties of Edible Mushrooms. In: Sridhar KR, Deshmukh SK (eds) Advances in Macrofungi: Industrial Avenues and Prospects. CRC Press, Boca Raton, pp 39–51
26. Khatua S, Paul S, Acharya K (2013) Mushroom as the potential source of new generation of antioxidant – A review. Res J Pharm Technol 6:496–505
27. Kües U, Badalyan SM (2017) In: Agrawal DC, Tsay HS, Shyur LF, Wu YC, Wang SY (eds) Making use of genomic information to explore the biotechnological potential of medicinal mushrooms. In: Medicinal plants and fungi: recent advances in research and development, Edition: Medicinal and Aromatic Plants of the World, vol 4. Springer Nature, pp 397–458
28. Lindequist U (2013) The merit of medicinal mushrooms from a pharmaceutical point of view. Int J Med Mushrooms 15:517–523
29. Lindequist U (2021) Macrofungi in pharmacy, medicine, cosmetics and nutrition - An appraisal. In: Sridhar KR, Deshmoukh SK (eds) Advances in Macrofungi: Pharmaceuticals and Cosmeceuticals. CRC Press, Boca Raton, pp 1–6
30. Mapoung S, Umsumarng S, Semmarath W, Arjsri P, Thippraphan P et al (2021) Skin wound-healing potential of polysaccharides from medicinal mushroom *Auricularia auricula-judae* (Bull.). J Fungi 7:247
31. Sánchez C (2017) Bioactives from mushrooms and their application. In: Puri M (ed) Food bioactives. Springer Nature, pp 23–57
32. Wasser SP (2014) Medicinal mushroom science: current perspectives, advances, evidences, and challenges. Biom J 37:345–356
33. Wasser SP (2017) Medicinal mushrooms in human clinical studies. Part I. Anticancer, oncoimmunological, and immunomodulatory activities: a review. Int J Med Mushrooms 19: 279–317
34. Yang YL, Tao QQ, Han JJ, Bao L, Liu HW (2017) Recent advance on bioactive compounds from the edible and medicinal fungi in China. In: Agrawal DC, Tsay HS, Shyur LF, Wu YC, Wang SY (eds) Medicinal plants and fungi: recent advances in research and development, Medicinal and aromatic plants of the world, vol 4, Springer Nature, pp 253–313
35. Chen B, Tian J, Zhang J, Wang K, Liu L et al (2017) Triterpenes and meroterpenes from *Ganoderma lucidum* with inhibitory activity against HMGs reductase, aldose reductase and α-glucosidase. Fitoterapia 120:6–16

36. Hapuarachchi KK, Cheng CR, Wen TC, Jeewon R, Kakumyan P (2017) Mycosphere Essays 20: Therapeutic potential of *Ganoderma* species: insights into its use as traditional medicine. Mycosphere 8:1653–1694
37. Klaus A, Kozarski M, Vunduk J, Petrović P, Nikšić M (2016) Antibacterial and antifungal potential of wild Basidiomycete mushroom *Ganoderma applanatum*. Lek Sirov 36:37–46
38. Ma HT, Hsieh JF, Chen ST (2015) Anti-diabetic effects of *Ganoderma lucidum*. Phytochemistry 114:109–113
39. Ma K, Ren J, Han J, Bao L, Li L et al (2014) Ganoboninketals A − C, antiplasmodial 3,4-seco-27-norlanostane triterpenes from *Ganoderma boninense*. Pat J Nat Prod 77:1847–1852
40. Peng X, Li L, Wang X, Zhu G, Li Z et al (2016) Antioxidant farnesylated hydroquinones from *Ganoderma capense*. Fitoterapia 111:18–23
41. Peng X, Liu J, Xia J, Wang C, Li X et al (2015) Lanostane triterpenoids from *Ganoderma hainanense* J.D. Zhao. Phytochem 114:137–145
42. Pu DB, Zheng X, Gao JB, Zhang XJ, Qi Y et al (2017) Highly oxygenated lanostane-type triterpenoids and their bioactivity from the fruiting body of *Ganoderma gibbosum*. Fitoterapia 119:1–7
43. Saltarelli R, Ceccaroli P, Buffalini M, Vallorani L, Casadei L et al (2015) Biochemical characterization, antioxidant and antiproliferative activities of different *Ganoderma* collections. J Mol Microbiol Biotechnol 25:16–25
44. Stojković DS, Barros L, Calhelha RC, Glamočlija J, Ćirić A et al (2014) A detailed comparative study between chemical and bioactive properties of *Ganoderma lucidum* from different origins. Int J Food Sci Nutr 65:42–47
45. Taofiq O, Heleno SA, Calhelha RC, Alves MJ, Barros L et al (2017) The potential of *Ganoderma lucidum* extracts as bioactive ingredients in topical formulations, beyond its nutritional benefits. Food Chem Toxicol 108:139–147
46. Wang K, Bao L, Xiong W, Ma K, Han J et al (2015) Lanostane triterpenes from the Tibetan medicinal mushroom *Ganoderma leucocontextum* and their inhibitory effects on HMG-CoA reductase and α-glucosidase. J Nat Prod 78:1977–1989
47. Xu H, Kong YY, Chen X, Guo MY, Bai XH et al (2016) Recombinant FIP-gat, a fungal immunomodulatory protein from *Ganoderma atrum*, induces growth inhibition and cell death in breast cancer cells. J Agric Food Chem 64:2690–2698
48. Zeng P, Guo Z, Zeng X, Hao C, Zhang Y et al (2018) Chemical, biochemical, preclinical and clinical studies of *Ganoderma lucidum* polysaccharide as an approved drug for treating myopathy and other diseases in China. J Cell Mol Med:1–20
49. Panthong S, Boonsathorn N, Chuchawankul S (2016) Antioxidant activity, anti-proliferative activity, and amino acid profiles of ethanolic extracts of edible mushrooms. Genet Mol Res 15(4)
50. Adebayo EA, Martínez-Carrera D, Morales P, Sobal M, Escudero H et al (2017) Comparative study of antioxidant and antibacterial properties of the edible mushrooms *Pleurotus levis, P. ostreatus, P. pulmonarius* and *P. tuber-regium*. Int J Food Sci Technol 53:1316–1330
51. Baskaran A, Chua KH, Sabaratnam V, Ram MR, Kuppusamy UR (2017) *Pleurotus giganteus* (Berk. Karun & Hyde), the giant Oyster mushroom inhibits NO production in LPS/H_2O_2 stimulated RAW 264.7 cells via STAT 3 and COX-2 pathways. BMC Complement Altern Med 17:1–10
52. Corrêa RC, Brugnari T, Bracht A, Peralta RM, Ferreira IC (2016) Biotechnological, nutritional and therapeutic uses of *Pleurotus* spp. (Oyster mushroom) related with its chemical composition: a review on the past decade findings. Trends Food Sci Technol 50:103–117
53. Finimundy TC, Dillon AJ, Henriques JA, Ely MR (2014) A review on general nutritional compounds and pharmacological properties of the *Lentinula edodes* Mushroom. Food Sci Nutr 5:1095–1105
54. Finimundy TC, Abreu RM, Bonetto N, Scariot FJ, Dillon AJ et al (2018) Apoptosis induction by *Pleurotus sajor-caju* (Fr.) Singer extracts on colorectal cancer cell lines. Food Chem Toxicol 112:383–392

55. Finimundy TC, Barros L, Calhelha RC, Alves MJ, Prieto MA et al (2018) Multifunctions of *Pleurotus sajor-caju* (Fr.) Singer: a highly nutritious food and a source for bioactive compounds. Food Chem 245:150–158
56. Fu Z, Liu Y, Zhang Q (2016) A potent pharmacological mushroom: *Pleurotus eryngii*. Fungal Genom Biol 6:139
57. Jayasuriya WB, Wanigatunge CA, Fernando GH, Abeytunga DT, Suresh TS (2015) Hypoglycaemic activity of culinary *Pleurotus ostreatus* and *P. cystidiosus* mushrooms in healthy volunteers and type 2 diabetic patients on diet control and the possible mechanisms of action. Phytother Res 29:303–309
58. Ma G, Yang W, Mariga AM, Fang Y, Ma N et al (2014) Purification, characterization and antitumor activity of polysaccharides from *Pleurotus eryngii* residue. Carbohydr Polym 114: 297–305
59. Masri HJ, Maftoun PM, Abd Malek R, Boumehira AZ, Pareek A et al (2017) The edible mushroom *Pleurotus* spp.: II. Medicinal values. Int J Biotech Well Indus 6:1–11
60. Patel Y, Naraian R, Singh VK (2012) Medicinal properties of *Pleurotus* species (Oyster Mushroom): a review. World J Fungal Plant Biol 3:1–12
61. Rahman MA, Rahman MS, Bin Bashir NM, Mia R, Hossain A et al (2021) Rationalization of mushroom-based preventive and therapeutic approaches to COVID-19: Review. Int J Med Mushrooms 23:1–11
62. Souilem F, Fernandes Â, Calhelha RC, Barreira JC, Barros L et al (2017) Wild mushrooms and their mycelia as sources of bioactive compounds: antioxidant, anti-inflammatory and cytotoxic properties. Food Chem 230:40–48
63. Tao QQ, Ma K, Bao L, Wang K, Han JJ et al (2016) New sesquiterpenoids from the edible mushroom *Pleurotus cystidiosus* and their inhibitory activity against α-glucosidase and PTP1B. Fitoterapia 111:29–35
64. Yen MT, Chang YH, Huang SJ, Cheng MC, Mau JL (2018) Extraction of ergothioneine from *Pleurotus eryngii* and *P. citrinopileatus* (Agaricomycetes) and preparation of its product. Int J Med Mushrooms 20:381–392
65. Zhang C, Li S, Zhang J, Hu C, Che G et al (2016) Antioxidant and hepatoprotective activities of intracellular polysaccharide from *Pleurotus eryngii* SI-04. Int J Biol Macromol 91:568–577
66. Zambonelli A, Bonito GM (2012) Edible ectomycorrhizal mushrooms, current knowledge and future prospects. Springer-Verlag, Berlin, p 409
67. Elmastas M, Turkekul I, Ozturk L, Gulcin I, Isildak O et al (2006) Antioxidant activity of two wild edible mushrooms (*Morchella vulgaris* and *Morchella esculenta*) from North Turkey. Comb Chem High Throughput Screen 9:443–448
68. Heleno SA, Stojković D, Barros L, Glamočlija J, Soković M et al (2013) A comparative study of chemical composition, antioxidant and antimicrobial properties of *Morchella esculenta* (L.) Pers. from Portugal and Serbia. Food Res Int 51:236–243
69. Ren F, Zhang Y, Yu H, Zhang YA (2020) *Ganoderma lucidum* cultivation affect microbial community structure of soil, wood segments and tree roots. Sci Rep 10:3435
70. Cerigini E, Palma F, Buffalini M, Amicucci A, Ceccaroli P et al (2007) Identification of a novel lectin from the Ascomycetes fungus *Tuber borchii*. Int J Med Mushrooms 9:287
71. Chang ST, Wasser SP (2012) The role of culinary-medicinal mushrooms on human welfare with a pyramid model for human health. Int J Med Mushrooms 14:95–134
72. Doğan HH, Aydin S (2013) Determination of antimicrobial effect, antioxidant activity and phenolic contents of desert truffle in Turkey. Afr J Trad Compl Alt Med 10:52–58
73. Murcia MA, Martinez-Tome M, Jimenez AM, Vera AM, Honrubia M et al (2002) Antioxidant activity of edible fungi (truffles and mushrooms): Losses during industrial processing. J Food Prot 65:1614–1622
74. Patel S, Rauf A, Khan H, Khalid S, Mubarak MS (2017) Potential health benefits of natural products derived from truffles: A review. Tr Food Sci Technol 70:1–8

75. Zhao W, Wang XH, Li HM, Wang SH, Chen T et al (2014) Isolation and characterisation of polysaccharides with the antitumor activity from *Tuber* fruiting bodies and fermentation system. Appl Microbiol Biotechnol 98:1991–2002
76. Stadler M, Hoffmeister D (2015) Fungal natural products – The mushroom perspective. Front Microbiol 6:127
77. Nielsen JC, Nielsen J (2017) Development of fungal cell factories for the production of secondary metabolites: linking genomics and metabolism. Synth Syst Biotechnol 2:5–12
78. Yap HY, Chooi YH, Firdaus-Raih M, Fung SY, Ng ST et al (2014) The genome of the tiger milk mushroom, *Lignosus rhinocerotis*, provides insights into the genetic basis of its medicinal properties. BMC Genomics 15:635
79. Yap HY, Fung SY, Ng ST, Tan CS, Tan NH (2015) Genome-based proteomic analysis of *Lignosus rhinocerotis* (Cooke) Ryvarden sclerotium. Int J Med Sci 12:23–31
80. Chang ST, Buswell JA (1996) Mushroom nutriceuticals. World J Microbiol Biotechnol 12: 473–476
81. Boa ER (2004) Wild edible fungi: A global overview of their use and importance to people. Food and agriculture organization of the United Nations, Rome
82. Li H, Tian Y, Menolli N Jr, Ye L, Karunarathna SC et al (2021) Reviewing the world's edible mushroom species: A new evidence-based classification system. Compr Rev Food Sci Food Saf 20:1982–2014
83. Li M, Hu JL (2014) Study on survival strategies of farmers engage in small-scale household cultivation of edible mushrooms: take shandong Province as an example. J Mod Econ 5:1092–1100
84. Royse DJ, Baars J, Tan Q (2017) Current overview of mushroom production in the world. In: Diego CZ, Pardo-Giménez AJ (eds) Edible and Medicinal Mushrooms: Technology and Applications. Wiley-Blackwell, pp 5–13
85. Vedder PJC, Den Munckhof-Vedder V (2020) Modern mushroom growing 2020. Harvesting:445
86. Zambonelli A, Iotti M, Boutahir S, Lancellotti E, Perini C et al (2012) Ectomycorrhizal fungal communities of edible ectomycorrhizal mushrooms. In: Zambonelli A, Bonito GM (eds) Edible ectomycorrhizal mushrooms, current knowledge and future prospects. Springer-Verlag, Berlin, pp 105–124
87. Sanmee R, Lumyong P, Dell B, Lumyong S (2010) In vitro cultivation and fruit body formation of the black bolete, *Phlebopus portentosus*, a popular edible ectomycorrhizal fungus in Thailand. Mycoscience 51:15–22
88. Zambonelli A, Iotti M, Puliga F, Hall IR (2021) Enhancing white truffle (*Tuber magnatum* Picco and *T. borchii* Vittad.) cultivation through biotechnology innovation. In: Al-Khayri JM, Jain SM, Johnson DV (eds) Advances in Plant Breeding Strategies: Vegetable Crops. Springer, Cham, pp 505–532
89. Dupont J, Dequin S, Giraud T, Le Tacon F, Marsit S et al (2017) Fungi as a source of food. ASM Journals, Microbiol Spectr 5(3)
90. González A, Cruz M, Losoya C, Nobre C, Loredo A et al (2020) Edible mushrooms as a novel protein source for functional foods. Food Funct 11:7400–7414
91. Kumar A, Singh M, Singh G (2013) Effect of different pretreatments on the quality of mushrooms during solar drying. J Food Sci Technol 50:165–170
92. Fernandes T, Garrine C, Ferrão J, Bell V, Varzakas T (2021) Mushroom nutrition as preventative healthcare in sub-Saharan Africa. Appl Sci 11:4221
93. He J, Evans NM, Liu H, Shao S (2020) A review of research on plant-based meat alternatives: Driving forces, history, manufacturing, and consumer attitudes. Compr Rev Food Sci Food Saf 19:2639–2656
94. Larpin C, Wozniak H, Genton L, Serratrice J (2019) Alimentations végétariennes et véganes: quelles conséquences sur la santé ? [Vegetarian and vegan diets and their impact on health]. Rev Med Suisse 15:1849–1853

95. Watanabe F, Yabuta Y, Bito T, Teng F (2014) Vitamin B_{12}-containing plant food sources for vegetarians. Nutrients 6:1861–1873
96. Diallo I, Boudard F, Morel S, Vitou M, Guzman C et al (2020) Antioxidant and anti-inflammatory potential of shiitake culinary-medicinal mushroom, *Lentinus edodes* (Agaricomycetes), sporophores from various culture conditions. Int J Med Mushrooms 22: 535–546
97. Usman M, Murtaza G, Ditta A (2021) Nutritional, Medicinal, and Cosmetic Value of Bioactive Compounds in Button Mushroom (*Agaricus bisporus*): A Review. Appl Sci 11:5943
98. Tietel Z, Masaphy S (2018) True morels (*Morchella*)-nutritional and phytochemical composition, health benefits and flavor: A review. Crit Rev Food Sci Nutr 58:1888–1901
99. Su S, Ding X, Fu L, Hou Y (2019) Structural characterization and immune regulation of a novel polysaccharide from Maerkang *Lactarius deliciosus* Gray. Int J Mol Med 44:713–724
100. Lee H, Nam K, Zahra Z, Farooqi MQU (2020) Potentials of truffles in nutritional and medicinal applications: a review. Fungal Biol Biotechnol 7:9
101. Bach C, Beacco P, Cammaletti P, Babel-Chen Z, Levesque E et al (2021) First production of Italian white truffle (*Tuber magnatum* Pico) ascocarps in an orchard outside its natural range distribution in France. Mycorrhiza 31:383–388
102. Oei P (2016) Mushroom Cultivation, 4th edn. Packhuys Publishers, Amsterdam, p 429
103. Sánchez C (2004) Modern aspects of mushroom culture technology. Appl Microbiol Biotechnol 64:756–762
104. Holm-Nielsen JB, Al Seadi T, Oleskowicz-Popiel P (2009) The future of anaerobic digestion and biogas utilization. Bioresour Technol 100:5478–5484
105. Lee M, Steiman M, Angelo S (2021) Biogas digestate as a renewable fertilizer: Effects of digestate application on crop growth and nutrient composition. Renew Agric Food Syst 36: 173–181
106. Czubaszek R, Wysocka-Czubaszek A (2018) Emissions of carbon dioxide and methane from fields fertilized with digestate from an agricultural biogas plant. Int Agrophys 32:29–37
107. Paolini V, Petracchini F, Segreto M, Tomassetti L, Naja N et al (2018) Environmental impact of biogas: A short review of current knowledge. J. Environ. Sci. Heal.-Part A Toxic/Hazard. Subst Environ Eng 53:899–906
108. Fornito S, Puliga F, Leonardi P, Di Foggia M, Zambonelli A et al (2020) Degradative ability of mushrooms cultivated on corn silage digestate. Molecules 25(13)
109. Brezáni A, Svobodova K, Jablonský I, Tlustoš P (2019) Cultivation of medicinal mushrooms on spruce sawdust fermented with a liquid digestate from biogas stations. Int J Med Mushrooms 21:215–223
110. Wang HW, Xu M, Cai XY, Tian F (2021) Evaluation of soil microbial communities and enzyme activities in cucumber continuous cropping soil treated with spent mushroom (*Flammulina velutipes*) substrate. J Soils Sediments 21:2938–2951
111. Wang HW, Xu M, Cai XY, Feng T, Xu WL (2020) Application of spent mushroom substrate suppresses *Fusarium* wilt in cucumber and alters the composition of the microbial community of the cucumber rhizosphere. Eur J Soil Biol 101:103245
112. Alhujaily A, Yu H, Zhang X, Ma F (2018) Highly efficient and sustainable spent mushroom waste adsorbent based on surfactant modification for the removal of toxic dyes. Int J Environ Res Public Health 15:1421
113. Gao X, Tang X, Zhao K, Balan V, Zhu Q (2021) Biogas production from anaerobic co-digestion of spent mushroom substrate with different livestock manure. Energies 14:570
114. Grimm D, Kuenz A, Rahmann G (2021) Integration of mushroom production into circular food chains. Org Agric 11:309–317
115. Grujić M, Dojnov B, Potočnik I, Duduk B, Vujčić Z (2015) Spent mushroom compost as substrate for the production of industrially important hydrolytic enzymes by fungi *Trichoderma* spp. and *Aspergillus niger* in solid state fermentation. Int Biodeterior Biodegrad 104:290–298

116. Liu Q, Ma H, Zhang Y, Dong C (2018) Artificial cultivation of true morels: current state, issues and perspectives. Crit Rev Biotechnol 38:259–271
117. Tan H, Liu T, Yu Y, Tang J, Jiang L (2021) Morel production related to soil microbial diversity and evenness. Microbiol Spectr 9:e00229–e00221
118. Sambyal K, Singh RV (2021) A comprehensive review on *Morchella importuna*: cultivation aspects, phytochemistry, and other significant applications. Folia Microbiol 66:147–157
119. Yuan B-H, Li H, Liu L, Du X-H (2021) Successful induction and recognition of conidiation, conidial germination and chlamydospore formation in pure culture of *Morchella*. Fungal Biol 125:285–293
120. Ori F, Hall I, Gianchino C, Iotti M, Zambonelli A (2019) Truffles and morels: two different evolutionary strategies of fungal-plant interactions in the Pezizales. In: Varma A, Tripathi S, Prasad R (eds) Plant Microbe Interface. Springer, Cham
121. Snabl M, Guidori U, Gianchino C, Iotti M, Zambonelli A (2019) Morels on the sand dunes of the Emilia-Romagna coast (Northwestern Adriatic Sea, Italy). Italian J Mycol 48:16–25
122. Raethong N, Wang H, Nielsen J, Vongsangnak W (2020) Optimizing cultivation of *Cordyceps militaris* for fast growth and cordycepin overproduction using rational design of synthetic media. Comput Struc Biotechnol J 18:1–8
123. Wua C-H, Liangb C-H, Liang Z-C (2021) Enhanced production of fruiting bodies and bioactive compounds of *Cordyceps militaris* with grain substrates and cultivation patterns. J Taiwan Inst Chem Eng. (in press)
124. Li X, Liu Q, Li W, Li Q, Qian Z et al (2021) Breakthrough in the artificial cultivation of Chinese cordyceps on a large-scale and its impact on science, the economy, and industry. Crit Rev Biotechnol 39:181–191
125. Elisashvili V (2012) Submerged cultivation of medicinal mushrooms: bioprocesses and products (review). Int J Med Mushrooms 14:211–239
126. Bakratsas G, Polydera A, Katapodis P, Stamatis H (2021) Recent trends in submerged cultivation of mushrooms and their application as a source of nutraceuticals and food additives. Future Foods 4:100086
127. Corrales A, Henkel TW, Smith ME (2018) Ectomycorrhizal associations in the tropics – biogeography, diversity patterns and ecosystem roles. New Phytol 220:1076–1091
128. McGuire KL, Allison SD, Fierer N, Treseder KK (2013) Ectomycorrhizal-dominated boreal and tropical forests have distinct fungal communities, but analogous spatial patterns across soil horizons. PLoS One 8:e68278
129. Hall I, Brown G, Zambonelli A (2007) Taming the Truffle. Lore, and Science of the Ultimate Mushroom, Timber Press, Portland, Oregon, The History, p 304
130. Iotti M, Piattoni F, Zambonelli A (2012) Techniques for host plant inoculation with truffles and other edible ectomycorrhizal mushrooms. In: Zambonelli A, Bonito GM (eds) Edible ectomycorrhizal mushrooms, current knowledge and future prospects, Soil biology, vol 34. Springer-Verlag, Berlin, pp 145–161
131. Zambonelli A, Rivetti C, Percurdani R, Ottonello S (2000) TuberKey: a DELTA-based tool for the description and interactive identification of truffles. Mycotaxon 74:57–76
132. Bonconpagni, S (2018). Disciplinare di produzione delle piante micorizzate con tartufo certificate. https://agricoltura.regione.emilia-romagna.it/fitosanitario/doc/Autorizzazioni/piante-micorrizate/piante-micorrizate
133. Donnini D, Benucci GMN, Bencivenga M, Baciarelli-Falini L (2014) Quality assessment of truffle-inoculated seedlings in Italy: proposing revised parameters for certification. For Syst 23:385–393
134. Murat C (2015) Forty years of inoculating seedlings with truffle fungi: past and future perspectives. Mycorrhiza 25:77–81
135. Selosse MA, Schneider-Maunoury L, Taschen E, Rousset F, Richard F (2017) Black truffle, a hermaphrodite with forced unisexual behavior. Trends Microbiol 25:784–787
136. Iotti M, Piattoni F, Leonardi P, Hall IR, Zambonelli A (2016) First evidence for truffle production from plants inoculated with mycelial pure cultures. Mycorrhiza 26:793–798

137. Leonardi P, Iotti M, Zeppa SD, Lancellotti E, Amicucci A et al (2017) Morphological and functional changes in mycelium and mycorrhizas of *Tuber borchii* due to heat stress. Fungal Ecol 29:20–29
138. Vahdatzadeh M, Splivallo R (2018) Improving truffle mycelium flavour through strain selection targeting volatiles of the Ehrlich pathway. Sci Rep 8:9304
139. Wang D, Zhang JL, Wang Y, Zambonelli A, Hall I et al (2021) The cultivation of *Lactarius* with edible mushrooms. Ital J Mycol 50:63–77
140. Chaudhuri S, Datta HK (2021) Macrofungal Polysaccharides as Immunoceuticals in Cancer Therapy. In: Sridhar KR, Deshmukh SK (eds) Advances in Macrofungi: Industrial Avenues and Prospects. CRC Press, Boca Raton, pp 287–310
141. Fernandes T, Garrine C, Ferrão J, Bell V, Varzakas T (2015) Chemical features of *Ganoderma* polysaccharides with antioxidant, antitumor and antimicrobial activities. Phytochemistry 114: 38–55
142. He X, Wang X, Fang J, Chang Y, Ning N et al (2017) Structures, biological activities, and industrial applications of the polysaccharides from *Hericium erinaceus* (Lion's mane) mushroom: a review. Int J Biol Macromol 97:228–237
143. He X, Wang X, Fang J, Chang Y, Ning N et al (2017) Polysaccharides in *Grifola frondosa* mushroom and their health promoting properties: a review. Int J Biol Macromol 101:910–921
144. Kozarski MS, Klaus A, Nikšić M, Van Griensven LJ, Vrvić M et al (2014) Polysaccharides of higher fungi: biological role, structure and antioxidative activity. Hem Ind 68:305–320
145. Meng X, Liang H, Luo L (2016) Antitumor polysaccharides from mushrooms: a review on the structural characteristics, antitumor mechanisms and immunomodulating activities. Carbohydr Res 424:30–41
146. Wang Q, Wang F, Xu Z, Ding Z (2017) Bioactive mushroom polysaccharides: a review on monosaccharide composition, biosynthesis and regulation. Molecules 22:955
147. Wang XL, Ding ZY, Zhao Y, Liu GQ, Zhou GY (2017) Efficient accumulation and *in vitro* antitumor activities of triterpene acids from submerged batch-cultured Lingzhi or Reishi medicinal mushroom, *Ganoderma lucidum* (Agaricomycetes). Int J Med Mushrooms 19: 419–431
148. Zhang M, Cui SW, Cheung PC, Wang Q (2007) Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. Trends Food Sci Technol 18:4–19
149. Zhang Y, Liu W, Xu C, Huang W, He P (2017) Characterization and antiproliferative effect of novel acid polysaccharides from the spent substrate of Shiitake culinary-medicinal mushroom *Lentinus edodes* (Agaricomycetes) cultivation. Int J Med Mushrooms 19:395–403
150. Zhu F, Du B, Bian Z, Xu B (2015) Beta-glucans from edible and medicinal mushrooms: characteristics, physicochemical and biological activities. J Food Compos Anal 41:165–173
151. Mironczuk-Chodakowska I, Witkowska AM, Zujko ME, Terlikowska KM (2017) Quantitative evaluation of 1,3-1,6-β-D-glucan contents in wild–growing species of edible Polish mushrooms. Rocz Panstw Zakl Hig 68:281–290
152. Subramaniam S, Raman J, Sabaratnam V, Heng CK, Kuppusamy UR (2017) Functional properties of partially characterized polysaccharide from the medicinal mushroom *Ganoderma neo-japonicum* (Agaricomycetes). Int J Med Mushrooms 19:849–859
153. Ferreira ICFR, Vaz JA, Vasconcelos MH, Martins A (2010) Compounds from wild mushrooms with antitumor potential. Anti Cancer Agents Med Chem 10:424–436
154. Islam T, Yu X, Xu B (2016) Phenolic profiles, antioxidant capacities and metal chelating ability of edible mushrooms commonly used in China. LWT Food Sci Technol 72:423–431
155. Kozarski M, Klaus A, Jakovljevic D, Todorovic N, Vunduk J et al (2015) Antioxidants of edible mushrooms. Molecules 20:19489–19525
156. Palacios I, Lozano M, Moro C, D'arrigo M, Rostagno MA et al (2011) Antioxidant properties of phenolic compounds occurring in edible mushrooms. Food Chem 128:674–678
157. Acharya K, Bera I, Khatua S, Rai M (2015) Pharmacognostic standardization of *Grifola frondosa*: a well-studied medicinal mushroom. Pharm Lett 7:72–78

158. Acharya K, Ghosh S, Kundu I (2016) Pharmacognostic standardization of a well-known edible mushroom, *Volvariella volvacea*. J App Pharm Sci 6:185–190
159. Buruleanu LC, Radulescu C, Georgescu AA, Danet FA, Olteanu RL et al (2018) Statistical characterization of the phytochemical characteristics of edible mushroom extracts. Anal Lett 51:1039–1059
160. Butkhup L, Samappito W, Jorjong S (2018) Evaluation of bioactivities and phenolic contents of wild edible mushrooms from northeastern Thailand. Food Sci Biotechnol 27:193–202
161. Nowacka N, Nowak R, Drozd M, Olech M, Los R et al (2014) Analysis of phenolic constituents, antiradical and antimicrobial activity of edible mushrooms growing wild in Poland. LWT – Food Sci Technol 59:689–694
162. Pop RM, Puia IC, Puia A, Chedea VS, Leopold N et al (2018) Characterization of *Trametes versicolor*: medicinal mushroom with important health benefits. Not Bot Horti Agrobo 46: 343–349
163. Reis FS, Heleno SA, Barros L, Sousa MJ, Martins A et al (2011) Toward the antioxidant and chemical characterization of mycorrhizal mushrooms from Northeast Portugal. J Food Sci 76: 824–830
164. Yao HM, Wang G, Liu YP, Rong MQ, Shen CB et al (2016) Phenolic acids isolated from the fungus *Schizophyllum commune* exerts analgesic activity by inhibiting voltage-gated sodium channels. Chin J Nat Med 14:661–670
165. Narsing Rao MP, Xiao M, Li WJ (2017) Fungal and Bacterial Pigments: Secondary Metabolites with Wide Applications. Front Microbiol 8:1113
166. Clericuzio M, Cassino C, Corana F, Vidari G (2012) Terpenoids from *Russula lepida* and *R. amarissima* (Basidiomycota, Russulaceae). Phytochemistry 84:154–159
167. Dasgupta A, Acharya K (2019) Mushrooms: an emerging resource for therapeutic terpenoids. 3 Biotech 9:369
168. Castellano G, Torrens F (2015) Information entropy-based classification of triterpenoids and steroids from *Ganoderma*. Phytochemistry 116:305–313. https://doi.org/10.1016/j.phytochem.2015.05.008
169. Duru ME, Çayan GT (2015) Biologically active terpenoids from mushroom origin: a review. Rec Nat Prod 9:456–483
170. Hadda M, Djamel C, Akila O (2015) Production and qualitative analysis of triterpenoids and steroids of *Ganoderma* species harvested from cork oak forest of North-Eastern Algeria. Res J Microbiol 10:366–376
171. Verekar SA, Gupta MK, Deshmukh SK (2021) *Fomitopsis betulina*: A Rich Source of Diverse Bioactive Metabolites. In: Sridhar KR, Deshmukh SK (eds) Advances in Macrofungi: Industrial Avenues and Prospects. CRC Press, Boca Raton, pp 52–68
172. Zhang SB, Li ZH, Stadler M, Chen HP, Huang Y et al (2018) Lanostane triterpenoids from *Tricholoma pardinum* with NO production inhibitory and cytotoxic activities. Phytochemistry 152:105–112
173. Zhao J, Yang Y, Yu M, Yao K, Luo X et al (2018) Lanostane-type triterpenoid derivatives from the fruiting bodies of cultivated *Fomitopsis palustris*. Phytochemistry 152:10–21
174. Zhao ZZ, Chen HP, Huang Y, Li ZH, Zhang L et al (2016) Lanostane triterpenoids from fruiting bodies of *Ganoderma leucocontextum*. Nat Prod Bioprospect 6:103–109
175. Yuyama KT, Fortkamp D, Abraham WR (2017) Eremophilane-type sesquiterpenes from fungi and their medicinal potential. Biol Chem 399:13–28
176. Isaka M, Sappan M, Supothina S, Srichomthong K, Komwijit S et al (2017) Alliacane sesquiterpenoids from submerged cultures of the Basidiomycete *Inonotus* sp. BCC 22670. Phytochemistry 136:175–181
177. Qiao X, Wang Q, Ji S, Huang Y, Zhang ZX et al (2015) Metabolites identification and multi-component pharmacokinetics of ergostane and lanostane triterpenoids in the anticancer mushroom *Antrodia cinnamomea*. J Pharm Biomed Anal 111:266–276
178. Tohtahon Z, Xue J, Han J, Liu Y, Hua H et al (2017) Cytotoxic lanostane triterpenoids from the fruiting bodies of *Piptoporus betulinus*. Phytochemistry 143:98–103

179. Sato M, Tai T, Nunoura Y, Yajima Y, Kawashima S et al (2002) Dehydrotrametenolic acid induces preadipocyte differentiation and sensitizes animal models of noninsulin-dependent diabetes mellitus to insulin. Biol Pharm Bull 25:81–86
180. Liu FY, Luo KW, Yu ZM, Co NN, Wu SH et al (2009) Suillin from the mushroom Suillus placidus as potent apoptosis inducer in human hepatoma HepG2 cells. Chem Biol Interact 181: 168–174
181. Shirata K, Kato T, Niwano M (1995) Selective suppression of the mitogenic response of murine lymphocytes by suillin from *Suillus bovines*. Mycologia 87:360–361
182. Hayashi T, Kanetoshi A, Ikura M, Shirhama H (1989) Bolegrevilol, a new lipid peroxidation inhibitor from the edible mushroom *Suillus grevillei*. Chem Pharm Bull 37:1424–1425
183. Ribeiro B, Lopes R, Andrade PB, Seabra RM, Gonçalves RF et al (2008) Comparative study of phytochemicals and antioxidant potential of wild edible mushroom caps and stipes. Food Chem 110:47–56
184. Kovacs B, Béni Z, Dékány M, Bózsity N, Zupko I et al (2018) Isolation and structure determination of antiproliferative secondary metabolites from the potato earthball mushroom, *Scleroderma bovista* (Agaricomycetes). Int J Med Mushrooms 20:411–418
185. Pemberton RT (1994) Agglutinins (lectins) from British higher fungi. Mycol Res 98:277–290
186. Wang HX, Ng TB, Ooi VEC (1998) Lectins from mushrooms – a review. Mycol Res 102:897–906
187. Wang HX, Ooi VEC, Ng TR, Chiu KW, Chang ST (1996) Hypotensive and vasorelaxing activities of a lectin from the edible mushroom *Tricholoma mongolicum*. Pharmacol Toxicol 79:318–323
188. Zhao S, Zhao Y, Li S, Zhao J, Zhang G et al (2010) A novel lectin with highly potent antiproliferative and HIV-1 reverse transcriptase inhibitory activities from the edible wild mushroom *Russula delica*. Glycoconj J 27:259–265
189. Zheng S, Li C, Ng TB, Wang HX (2007) A lectin with mitogenic activity from the edible wild mushroom *Boletus edulis*. Process Biochem 42:1620–1624
190. Wu Y, Wang H, Ng TB (2011) Purification and characterization of a lectin with antiproliferative activity toward cancer cells from the dried fruit bodies of *Lactarius flavidulus*. Carbohydr Res 346:2576–2581
191. Antonyuk VO, Klyuchivska OY, Stoika RS (2010) Cytotoxic proteins of *Amanita virosa* Secr. mushroom: purification, characteristics and action towards mammalian cells. Toxicon 55: 1297–1305
192. Bovi M, Carrizo ME, Capaldi S, Perduca M, Chiarelli LR et al (2011) Structure of a lectin with antitumoral properties in king bolete (*Boletus edulis*) mushrooms. Glycobiology 21:1000–1009
193. Liu Q, Wang H, Ng TB (2004) Isolation and characterization of a novel lectin from the wild mushroom *Xerocomus spadiceus*. Peptides 25:7–10
194. Horibe M, Kobayashi Y, Dohra H, Morita T, Murata T et al (2011) Toxic isolectins from the mushroom Boletus venenatus. Phytochemistry 71:648–657
195. Lyimo B, Yagi F, Minami Y (2011) Primary structure and specificity of a new member of galectin family from the amethyst deceiver mushroom *Laccaria amethystina*. Biosci Biotechnol Biochem 75:62–69
196. Wälti MA, Walser PJ, Thore S, Grünler A, Bednar M et al (2008) Structural basis for chitotetraose coordination by CGL3, a novel galectin-related protein from *Coprinopsis cinerea*. J Mol Biol 379:146–159
197. Chen H, Tian T, Miao H, Zhao YY (2016) Traditional uses, fermentation, phytochemistry and pharmacology of *Phellinus linteus*: a review. Fitoterapia 113:6–26
198. Chen JT, Tominaga K, Sato Y, Anzai H, Matsuoka R (2010) Maitake mushroom (*Grifola frondosa*) extract induces ovulation in patients with polycystic ovary syndrome: a possible monotherapy and a combination therapy after failure with first-line clomiphene citrate. J Altern Complement Med 16:1295–1299

199. Scarpari M, Parroni A, Zaccaria M, Fattorini L, Bello C et al (2016) *Trametes versicolor* bioactive compounds stimulate *Aspergillus flavus* antioxidant system and inhibit aflatoxin synthesis. Plant Biosyst 150:653–659
200. Vetter J (2021) Mushrooms as Functional Foods. In: Sridhar KR, Deshmukh SK (eds) Advances in Macrofungi: Industrial Avenues and Prospects. CRC Press, Boca Raton, pp 139–174
201. Khan M, Tania M, Zhang D, Chen H (2010) *Cordyceps* mushroom: a potent anticancer nutraceutical. Open Nutraceuticals J 3:179–183
202. Xu J, Huang Y, Chen XX, Zheng SC, Chen P et al (2016) The mechanisms of pharmacological activities of *Ophiocordyceps sinensis* fungi. Phytother Res 30:1572–1583
203. Yang H, Yin T, Zhang S (2015) Isolation, purification, and characterisation of polysaccharides from wide *Morchella esculenta* (L.) Pers. Int J Food Prop 18:1385–1390
204. Chepkirui C, Yuyama KT, Wanga LA, Decock C, Matasyoh JC et al (2018) Microporenic acids A-G, biofilm inhibitors, and antimicrobial agents from the basidiomycete *Microporus* species. J Nat Prod 81:778–784
205. Doğan HH, Karagöz S, Duman R (2018) *In vitro* evaluation of the antiviral activity of some mushrooms from Turkey. Int J Med Mushrooms 20:201–212
206. Gargano ML, van Griensven LJ, Isikhuemhen OS, Lindequist U, Venturella G et al (2017) Medicinal mushrooms: valuable biological resources of high exploitation potential. Plant Biol 151:548–565
207. Krupodorova T, Rybalko S, Barshteyn V (2014) Antiviral activity of Basidiomycete mycelia against influenza type A (serotype H_1N_1) and herpes simplex virus type 2 in cell culture. Virol Sin 29:284–290
208. Morris HJ, Beltrán Y, Llauradó G, Batista PL, Perraud-Gaime I et al (2017) Mycelia from *Pleurotus* sp. (Oyster mushroom): a new wave of antimicrobials, anticancer and antioxidant bio-ingredients. Intl J Phyto Natur Ingrd 4:03
209. Santoyo S, Ramirez AC, Garcia LA, Reglero G, Soler-rivas C (2012) Antiviral activities of *Boletus edulis*, *Pleurotus ostreatus* and *Lentinus edodes* extracts and polysaccharide fractions against Herpes simplex virus type 1. J Food Nutr Res 51:225–235
210. Shen HS, Shao S, Chen JC, Zhou T (2017) Antimicrobials from mushrooms for assuring food safety. Compr Rev Food Sci Food Saf 16:316–329
211. Teplyakova TV, Kosogova TA (2016) Antiviral effect of Agaricomycetes mushrooms (Review). Int J Med Mushrooms 18:375–386
212. Waithaka PN, Gathuru EM, Githaiga BM, Onkoba KM (2017) Antimicrobial activity of mushroom (*Agaricus bisporus*) and fungal (*Trametes gibbosa*) extracts from mushrooms and fungi of Egerton Main Campus, Njoro Kenya. J Biomedical Sci 6:20
213. Owaid MN, Al Saeedi SSS, Ali Abed I, Shahbazi P, Sabaratnam V (2017) Antifungal activities of some *Pleurotus* species (higher Basidiomycetes). WJST 14:215–224
214. Shang X, Tan Q, Liu R, Yu K, Li P et al (2013) *In vitro* anti-*Helicobacter pylori* effects of medicinal mushroom extracts, with special emphasis on the Lion's mane mushroom, *Hericium erinaceus* (Higher Basidiomycetes). Int J Med Mushrooms 15:165–174
215. Schillaci D, Cusimano MG, Cascioferro SM, Di Stefano V, Arizza V et al (2017) Antibacterial activity of desert truffles from Saudi Arabia against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Int J Med Mushrooms 19:121–125
216. Abdullah N, Haimi MZ, Lau BF, Annuar MS (2013) Domestication of a wild medicinal sclerotial mushroom, *Lignosus rhinocerotis* (Cooke) Ryvarden. Ind Crop Prod 47:256–261
217. Ellan K, Sabaratnam V & Thayan R (2013). Antiviral activity and mode of action of mushroom extracts against dengue virus type-2. In: Proceedings of the 3rd international conference on dengue and dengue haemorrhagic fever, 21–23 October 2012, Bangkok.
218. Teplyakova TV, Psurtseva NV, Kosogova TA, Mazurkova NA, Khanin VA et al (2012) Antiviral activity of polyporoid mushrooms (Higher Basidiomycetes) from Altai Mountains (Russia). Int J Med Mushrooms 14:37–45

219. Mohiuddin AK (2021) Can medical mushrooms fight against Sars-Cov-2/Covid-19? J Internal Med Sci Art 2:23–24
220. Murphy EJ, Masterson C, Rezoagli E, O'Toole D, Major I et al (2020) β-Glucan extracts from the same edible shiitake mushroom *Lentinus edodes* produce differential in-vitro immunomodulatory and pulmonary cytoprotective effects - Implications for coronavirus disease (COVID-19) immunotherapies. Sci Total Environ 732:139330
221. Teplyakova TV, Ilyicheva TN, Kosogova TA, Wasser SP (2021) Medicinal mushrooms against Influenza viruses. Int J Med Mushhrooms 23:1–11
222. Hetland G, Johnson E, Bernardshaw SV, Grinde B (2021) Can medicinal mushrooms have prophylactic or therapeutic effect against COVID-19 and its pneumonic superinfection and complicating inflammation? Scand J Immunol 93:e12937
223. Brendler T, Al-Harrasi A, Bauer R, Gafner S, Hardyet ML et al (2021) Botanical drugs and supplements affecting the immune response in the time of COVID-19: Implications for research and clinical practice. Phytother Res 35:3013–3031
224. Shahzad F, Anderson D, Najafzadeh M (2020) The antiviral, anti-inflammatory effects of natural medicinal herbs and mushrooms and SARS-CoV-2 infection. Nutrients 12:2573
225. Del Buono A, Bonucci M, Pugliese S, D'orta A, Fioranelli M (2016) Polysaccharide from *Lentinus edodes* for integrative cancer treatment: immunomodulatory effects on lymphocyte population. WCRJ 3:1–7
226. Diling C, Chaoqun Z, Jian Y, Jian L, Jiyan S et al (2017) Immunomodulatory activities of a fungal protein extracted from *Hericium erinaceus* through regulating the gut microbiota. Front Immunol 8:666
227. Rubel R, Dalla Santa HS, Dos Santos LF, Fernandes LC, Figueiredo BC et al (2018) Immunomodulatory and antitumoral properties of *Ganoderma lucidum* and *Agaricus brasiliensis* (Agaricomycetes) medicinal mushrooms. Int J Med Mushrooms 20:393–403
228. Wang Y, Tian Y, Shao J, Shu X, Jia J et al (2018) Macrophage immunomodulatory activity of the polysaccharide isolated from *Collybia radicata* mushroom. Int J Biol Macromol 108:300–306
229. Guggenheim AG, Wright KM, Zwickey HL (2014) Immune modulation from five major mushrooms: application to integrative oncology. Integr Med 13:32–41
230. Khan MS, Zhang X, You L (2014) Structure and bioactivities of fungal polysaccharides. In: Ramawat K, Mérillon JM (eds) Polysaccharides-Bioactivity and Biotechnology. Springer, Cham, pp 1851–1866
231. Pandya U, Dhuldhaj U, Sahay NS (2018) Bioactive mushroom polysaccharides as antitumor: an overview. Nat Prod Res 4:1–13
232. Tsai MY, Hung YC, Chen YH, Chen YH, Huang YC et al (2016) A preliminary randomised controlled study of short-term *Antrodia cinnamomea* treatment combined with chemotherapy for patients with advanced cancer. BMC Complement Altern Med 16:322
233. Jiang J, Sliva D (2010) Novel medicinal mushroom blend suppresses growth and invasiveness of human breast cancer cells. Int J Oncol 37:1529–1536
234. Wesa KM, Cunningham-Rundles S, Klimek VM, Vertosick E, Coleton MI et al (2015) Maitake mushroom extract in myelodysplastic syndromes (MDS): a phase II study. Cancer Immunol Immunother 64:237–247
235. Alonso EN, Ferronato MJ, Fermento ME, Gandini NA, Romero AL et al (2018) Antitumoral and antimetastatic activity of Maitake D-Fraction in triple-negative breast cancer cells. Oncotarget 9:23396–23412
236. Rios JL, Andujar I, Recio MC, Giner RM (2012) Lanostanoids from fungi: a group of potential anticancer compounds. J Nat Prod 75:2016–2044
237. Da Silva MS, Smiderle FR, Biscaia SM, Rosado FR, Trindade ES et al (2018) Fucogalactan from the giant mushroom *Macrocybe titans* inhibits melanoma cells migration. Carbohydr Polym 190:50–56

238. Barbieri A, Quagliariello V, Del Vecchio V, Falco M, Luciano A et al (2017) Anticancer and anti-inflammatory properties of *Ganoderma lucidum* extract effects on melanoma and triple-negative breast cancer treatment. Nutrients 9:210
239. Welti S, Moreau PA, Azaroual N, Lemoine A, Duhal N et al (2010) Antiproliferative activities of methanolic extracts from a neotropical *Ganoderma* species (Aphyllophoromycetideae): identification and characterization of a novel ganoderic acid. Int J Med Mushrooms 12:17–31
240. Lemieszek MK, Nunes FH, Sawa-Wejksza K, Rzeski W (2017) A King Bolete, *Boletus edulis* (Agaricomycetes), RNA fraction stimulates proliferation and cytotoxicity of natural killer cells against myelogenous leukemia cells. Int J Med Mushrooms 19:347–353
241. Awadasseid A, Hou J, Gamallat Y, Xueqi S, Eugene KD et al (2017) Purification, characterization, and antitumor activity of a novel glucan from the fruiting bodies of *Coriolus versicolor*. PLoS One 12:e0171270
242. Gründemann C, Arnhold M, Meier S, Bäcker C, Garcia-Käufer M et al (2016) Effects of *Inonotus hispidus* extracts and compounds on human immunocompetent cells. Planta Med 82: 1359–1367
243. Gao Y, Padhiar AA, Wang J, Zhang W, Zhong M et al (2018) Recombinant latcripin 11 of *Lentinula edodes* C91-3 suppresses the proliferation of various cancer cells. Gene 642:212–219
244. Ebrahimi A, Atashi A, Soleimani M, Mashhadikhan M, Barahimi A et al (2017) Anti-invasive and antiproliferative effects of *Pleurotus ostreatus* extract on acute leukemia cell lines. J Basic Clin Physiol Pharmacol 29:95–102
245. Yang H, Sun W, Zhang J, Zhang Y, Yang Y et al (2018) Autophagy inhibition enhances SPCA-1 cell proliferation inhibition induced by By-1 from the stout camphor medicinal mushroom, *Taiwanofungus camphoratus* (Agaricomycetes). Int J Med Mushrooms 20:321–335
246. Aras A, Khalid S, Jabeen S, Farooqi AA, Xu B (2018) Regulation of cancer cell signaling pathways by mushrooms and their bioactive molecules: overview of the journey from bench-top to clinical trials. Food Chem Toxicol 119:206–214
247. Anwar H, Hussain G, Mustafa I (2018) Antioxidants from natural sources. In: Shalaby E, Mostafa GM (eds) Antioxidants in foods and its applications. IntechOpen, pp 3–28
248. Bandara AR, Karunarathna SC, Mortimer PE, Hyde KD, Khan S et al (2017) First successful domestication and determination of nutritional and antioxidant properties of the red ear mushroom *Auricularia thailandica* (Auriculariales, Basidiomycota). Mycol Prog 16:1029–1039
249. Carocho M, Ferreira IC, Morales P, Soković M (2018) Antioxidants and pro-oxidants: effects on health and aging. Oxidative Med Cell Longev 2018:ID1472708
250. Choi YJ, Park IS, Kim MH, Kwon B, Choo YM et al (2019) The medicinal mushroom *Auricularia auricula-judae* (Bull.) extract has antioxidant activity and promotes procollagen biosynthesis in HaCaT cells. Nat Prod Res 33:3283–3286
251. Ćilerdžić J, Kosanic M, Stajić M, Vukojević J, Ranković B (2016) Species of genus *Ganoderma* (Agaricomycetes) fermentation broth: a novel antioxidant and antimicrobial agent. Int J Med Mushrooms 18:397–404
252. Silva D, de Souza AC, de Almeida GG, Soares AA, de Sá-Nakanishi AB, de Santi-Rampazzo AP et al (2018) The antioxidant action of an aqueous extract of royal sun medicinal mushroom, *Agaricus brasiliensis* (Agaricomycetes) in rats with adjuvant-induced arthritis. Int J Med Mushrooms 20:101–117
253. Debnath S, Upadhyay RC, Das P, Saha AK (2017) Antioxidant activities of methanolic extracts from ten *Pleurotus* species. Int Res J Pharm 8:44–49
254. Heleno SA, Ferreira RC, Antonio AL, Queiroz MJ, Barros L et al (2015) Nutritional value, bioactive compounds and antioxidant properties of three edible mushrooms from Poland. Food Biosci 11:48–55
255. Ke L, Chen H (2016) Homogenate extraction of crude polysaccharides from *Lentinus edodes* and evaluation of the antioxidant activity. Food Sci Technol 36:533–539

256. Khaskheli AA, Khaskheli SG, Liu Y, Sheikh SA, Wang YF et al (2018) Characterization and antioxidant properties of crude water soluble polysaccharides from three edible mushrooms. J Med Plant Res 12:133–138
257. Khatun S, Islam A, Cakilcioglu U, Guler P, Chatterjee NC (2015) Nutritional qualities and antioxidant activity of three edible Oyster mushrooms (*Pleurotus* spp.). NJAS - Wageningen J Life Sci 72–73:1–5
258. Morel S, Arnould S, Vitou M, Boudard F, Guzman C et al (2018) Antiproliferative and antioxidant activities of wild Boletales mushrooms from France. Int J Med Mushrooms 20:13–29
259. Taofiq O, González-Paramás AM, Martins A, Barreiro MF, Ferreira IC (2016) Mushrooms extracts and compounds in cosmetics, cosmeceuticals and nutricosmetics – A review. Ind Crop Prod 90:38–48
260. Taofiq O, Heleno SA, Calhelha RC, Alves MJ, Barros L et al (2016) Development of mushroom-based cosmeceutical formulations with anti-inflammatory, anti-tyrosinase, antioxidant, and antibacterial properties. Molecules 21:1372
261. Vasdekis EP, Karkabounas A, Giannakopoulos I (2018) Screening of mushrooms bioactivity: piceatannol was identified as a bioactive ingredient in the order Cantharellales. Eur Food Res Technol 244:861–871
262. Kushairi N, Phan CW, Sabaratnam V, David P, Naidu M (2019) Lion's mane mushroom, *Hericium erinaceus* (Bull.: Fr.) Pers. suppresses H_2O_2-induced oxidative damage and LPS-induced inflammation in HT22 hippocampal neurons and BV2 microglia. Antioxidants 8:261
263. Novakovic A, Karaman M, Kaisarevic S, Radusin T (2017) Antioxidant and antiproliferative potential of fruiting bodies of the wild-growing king bolete mushroom, *Boletus edulis* (Agaricomycetes), from Western Serbia. Int J Med Mushrooms 19:27–34
264. Sánchez C (2017) Reactive oxygen species and antioxidant properties from mushrooms. Synth Syst Biotechnol 2:13–22
265. Zhu Y, Yu X, Ge Q, Li J, Wang D et al (2020) Antioxidant and antiaging activities of polysaccharides from *Cordyceps cicadae*. Int J Biol Macromol 157:394–400
266. Ruthes AC, Carbonero ER, Córdova MM, Baggio CH, Santos AR et al (2013) *Lactarius rufus* (1-3)-, (1-6)-β-D-glucans: structure, antinociceptive and anti-inflammatory effects. Carbohydr Polym 94:129–136
267. Ahn H, Jeon E, Kim JC, Kang SG, Yoon SI et al (2017) Lentinan from Shiitake selectively attenuates AIM2 and non-canonical inflammasome activation while inducing pro-inflammatory cytokine production. Sci Rep 7:1314
268. Nallathamby N, Phan CW, Seow SL, Baskaran A, Lakshmanan H et al (2018) A status review of the bioactive activities of tiger milk mushroom *Lignosus rhinocerotis* (Cooke) Ryvarden. Front Pharmacol 8:998
269. Martel J, Ojcius DM, Chang CJ, Lin CS, Lu CC et al (2017) Anti-obesogenic and antidiabetic effects of plants and mushrooms. Nat Rev Endocrinol 13:149–161
270. Morales D, Piris AJ, Ruiz-Rodriguez A, Prodanov M, Soler-Rivas C (2018) Extraction of bioactive compounds against cardiovascular diseases from *Lentinula edodes* using a sequential extraction method. Biotechnol Prog 34:746–755
271. Vitak T, Yurkiv B, Wasser S, Nevo E, Sybirna N (2017) Effect of medicinal mushrooms on blood cells under conditions of diabetes mellitus. World J Diabetes 8:187–201
272. Hong L, Xun M, Wutong W (2007) Anti-diabetic effect of an alpha-glucan from fruit body of Maitake (*Grifola frondosa*) on KK-Ay mice. J Pharm Pharmacol 59:575–582
273. Kim YW, Kim KH, Choi HJ, Lee DS (2005) Anti-diabetic activity of β-glucans and their enzymatically hydrolyzed oligosaccharides from *Agaricus blazei*. Biotechnol Lett 27:483–487
274. Thongbai B, Rapior S, Hyde KD, Wittstein K, Stadler M (2015) *Hericium erinaceus*, an amazing medicinal mushroom. Mycol Prog 14:91
275. Yamaç M, Kanbak G, Zeytinoglu M, Senturk H, Bayramoglu G et al (2010) Pancreas protective effect of button mushroom *Agaricus bisporus* (J.E. Lange) Imbach

(Agaricomycetidae) extract on rats with Streptozotocin-induced diabetes. Int J Med Mushrooms 12:379–389
276. Yamaç M, Zeytinoglu M, Senturk H, Kartkaya K, Kanbak G et al (2016) Effects of black hoof medicinal mushroom, *Phellinus linteus* (Agaricomycetes), polysaccharide extract in streptozotocin-induced diabetic rats. Int J Med Mushrooms 18:301–311
277. Afrin S, Rakib MA, Kim BH, Kim JO, Ha YL (2016) Eritadenine from edible mushrooms inhibits activity of angiotensin converting enzyme *in vitro*. J Agric Food Chem 64:2263–2268
278. Ehsanifard Z, Mir-Mohammadrezaei F, Safarzadeh A, Ghobad-Nejhad M (2017) Aqueous extract of *Inocutis levis* improves insulin resistance and glucose tolerance in high sucrose-fed Wistar rats. J Herbmed Pharmacol 6:160–164
279. Paravamsivam P, Heng CK, Malek SN, Sabaratnam V, Kuppusamy UR (2016) Giant Oyster mushroom *Pleurotus giganteus* (Agaricomycetes) enhances adipocyte differentiation and glucose uptake via activation of PPAR and glucose transporters 1 and 4 in 3T3-L1 cells. Int J Med Mushrooms 18:821–831
280. Singh V, Bedi GK, Shri R (2017) *In vitro* and *in vivo* antidiabetic evaluation of selected culinary-medicinal mushrooms (Agaricomycetes). Int J Med Mushrooms 19:17–25
281. Gil-Ramirez A, Morales D, Soler-Rivas C (2017) Molecular actions of hypocholesterolaemic compounds from edible mushrooms. Food Funct 9:53–69
282. Khatun K, Mahtab H, Khanam PA, Sayeed MA, Khan KA (2007) Oyster mushroom reduced blood glucose and cholesterol in diabetic subjects. Mymensingh Med J 16:94–99
283. Khatun S, Islam A, Cakilcioglu U, Chatterjee NC (2012) Research on mushroom as a potential source of nutraceuticals: a review on Indian perspective. Am J Experim Agricult 2:47–73
284. Shibu MA, Agrawal DC, Huang CY (2017) Mushrooms: a Pandora box of cardioprotective phytochemicals. In: Agrawal DC, Tsay HS, Shyur LF, Wu YC, Wang SY (eds) Medicinal plants and fungi: recent advances in research and development, Edition: Medicinal and aromatic plants of the world, vol 4. Springer Nature, pp 337–362
285. Sabaratnam V, Kah-Hui W, Naidu M, David PR (2013) Neuronal health - can culinary and medicinal mushrooms help? J Tradit Complement Med 3:62–68
286. Yadav SK, Ir R, Jeewon R, Doble M, Hyde KD et al (2020) A mechanistic review on medicinal mushrooms-derived bioactive compounds: potential mycotherapy candidates for alleviating neurological disorders. Planta Med 86:1–15
287. Brandalise F, Cesaroni V, Gregori A, Repetti M, Romano C et al (2017) Dietary supplementation of *Hericium erinaceus* increases mossy fiber-CA3 hippocampal neurotransmission and recognition memory in wild-type mice. Evid Based Complement Alternat Med 2017:3864340
288. Chiu CH, Chyau CC, Chen CC, Lee LY, Chen WP et al (2018) Erinacine A-enriched *Hericium erinaceus* mycelium produces antidepressant-like effects through modulating BDNF/PI3K/Akt/GSK-3 signaling in mice. Int J Mol Sci 19:341
289. Rupcic Z, Rascher M, Kanaki S, Köster RW, Stadler M et al (2018) Two new cyathane diterpenoids from mycelial cultures of the medicinal mushroom *Hericium erinaceus* and the rare species, *Hericium flagellum*. Int J Mol Sci 19:740
290. Wittstein K, Rascher M, Rupcic Z, Löwen E, Winter B et al (2016) Corallocins A−C, nerve growth and brain-derived neurotrophic factor inducing metabolites from the mushroom *Hericium coralloides*. J Nat Prod 79:2264–2269
291. Carhart-Harris RL, Roseman L, Bolstridge M, Demetriou L, Pannekoek JN et al (2017) Psilocybin for treatment-resistant depression: fMRI-measured brain mechanisms. Sci Rep 7:1–11
292. Donatini B (2011) Hericium erinaceus: properties mostly related to the secretion of neuronal growth factor. Phytothérapie-Heidelberg 9:48–52
293. Phan CW, David P, Naidu M, Wong KH, Sabaratnam V (2015) Therapeutic potential of culinary-medicinal mushroom for the management of neurodegenerative diseases: diversity, metabolite, and mechanism. Crit Rev Biotechnol 35:355–368
294. Phan CW, David P, Sabaratnam V (2017) Edible and medicinal mushrooms: emerging brain food for the mitigation of neurodegenerative diseases. J Med Food 20:1–10

295. Phan CW, Tan EYY, Sabaratnam V (2018) Bioactive molecules in edible and medicinal mushrooms for human wellness. In: Mérillon JM, Ramavath K, Dhanasekara M (eds) Bioactive molecules in food. Reference series in phytochemistry. Springer, Cham, pp 1–24
296. Wong KH, Ng CC, Kanagasabapathy G, Yow YY, Sabaratnam V (2017) An overview of culinary and medicinal mushrooms in neurodegeneration and neurotrauma research. Int J Med Mushrooms 19:191–202
297. Seow SL, Eik LF, Naidu M, David P, Wong KH et al (2015) *Lignosus rhinocerotis* (Cooke) Ryvarden mimics the neuritogenic activity of nerve growth factor via MEK/ERK1/2 signaling pathway in PC-12 cells. Sci Rep 5:1–13
298. Seow SL, Naidu M, David P, Wong KH, Sabaratnam V (2013) Potentiation of neuritogenic activity of medicinal mushrooms in rat pheochromocytoma cells. BMC Complement Altern Med 13:157
299. Tsuk S, Lev YH, Rotstein A, Zeev A, Carasso R et al (2018) Effects of a commercial supplement of *Ophiocordyceps sinensis* and *Ganoderma lucidum* on physiological responses to maximal exercise in healthy young participants. Int J Med Mushrooms 20:359–367
300. Sabaratnam V, Phan CW (2017) Neuroactive components of culinary and medicinal mushrooms with potential to mitigate age-related neurodegenerative diseases. In: Brahmachari G (ed) Discovery and development of neuroprotective agents from natural products, 1st edn. Elsevier, pp 401–413
301. Gupta S, Summuna B, Gupta M, Annepu SK (2018) Edible mushrooms: cultivation, bioactive molecules, and health benefits. In: Mérillon JM, Ramawat KG (eds) Bioactive molecules in food, Reference Series in Phytochemistry. Springer-Verlag, pp 1–33
302. Živkovíc J, Ivanov M, Stojkovíc D, Glamčlija J (2021) Ethnomycological investigation in Serbia: astonishing realm of mycomedicines and mycofood. J Fungi 7:349
303. Chandrawanshi NK, Tandia DK, Jadhav SK (2017) Nutraceutical properties evaluation of *Schizophyllum commune*. Indian J Sci Res 13:57–62
304. Dhakal S, Kushairi N, Phan CW, Adhikari B, Sabaratnam V et al (2019) Dietary polyphenols: a multifactorial strategy to target Alzheimer's disease. Int J Mol Sci 20:5090
305. Ho LH, Zulkifli NA, Tan TC (2020) Edible mushroom: nutritional properties, potential nutraceutical values, and its utilization in food product development. An introduction to mushroom. IntechOpen, In
306. Rossi P, Difrancia R, Quagliariello V, Savino E, Tralongo P et al (2018) B-glucans from *Grifola frondosa* and *Ganoderma lucidum* in breast cancer: an example of complementary and integrative medicine. Oncotarget 9:24837–24856
307. Khatua S, Ghosh S, Acharya K (2017) *Laetiporus sulphureus* (Bull.:Fr.) Murr. as food as medicine. Pharmacogn J 9:1–15
308. Raman J, Jang KY, Hyun-Jae S, Kong WS, Lakshmanan H (2021) Industrial and biotechnological applications of *Pleurotus*. In: Sridhar KR, Deshmukh SK (eds) Advances in Macrofungi: Industrial Avenues and Prospects. CRC Press, Boca Raton, pp 89–107
309. Ahad S, Tanveer S, Malik TA (2016) Anticoccidial activity of aqueous extract of a wild mushroom (*Ganoderma applanatum*) during experimentally induced coccidial infection in broiler chicken. J Parasit Dis 40:408–414
310. Atila F, Owaid MN, Shariati MA (2017) The nutritional and medical benefits of *Agaricus bisporus*: a review. J Microbiol Biotechnol Food Sci 7:281–286
311. Azelee NI, Manas NH, Dailin DJ, Malek R, Moloi N et al (2021) Mushroom Bioactive Ingredients in Cosmetic Industries. In: Sridhar KR, Deshmukh SK (eds) Advances in Macrofungi: Industrial Avenues and Prospects. CRC Press, Boca Raton, pp 207–229
312. Barros L, Cruz T, Baptista P (2008) Wild and commercial mushrooms as source of nutrients and nutraceuticals. Food Chem Toxicol 46:2742–2747
313. Chen W, Zhao Z, Chen SF, Li YQ (2008) Optimization for the production of exopolysaccharide from *Fomes fomentarius* in submerged culture and its antitumor effect in vitro. Bioresour Technol 99:3187–3194

314. Chuang WY, Hsieh YC, Lee TT (2020) The effects of fungal feed additives in animals: a review. Animals (Basel) 10:805
315. Corrêa RC, Barros L, Fernandes Â, Sokovic M, Bracht A et al (2018) A natural food ingredient based on ergosterol: optimization of the extraction from *Agaricus blazei*, evaluation of bioactive properties and incorporation in yogurts. Food Funct 9:1465–1474
316. Dissanayake AA, Zhang CR, Mills GL, Nair MG (2018) Cultivated Maitake mushroom demonstrated functional food quality as determined by in vitro bioassays. J Funct Foods 44: 79–85
317. Dutta S (2013) Role of mushrooms as nutraceutical an overview. Int J Pharm Bio Sci 4:B59–B66
318. El-Shazly KA, El-Latif AA, Abdo W, El-Morsey A, El-Aziz MI et al (2020) The anticoccidial activity of the fluoroquinolone lomefloxacin against experimental *Eimeria tenella* infection in broiler chickens. Parasitol Res 119:1955–1968
319. Farzana T, Mohajan S, Saha T, Hossain MN, Haque MZ (2017) Formulation and nutritional evaluation of a healthy vegetable soup powder supplemented with soy flour, mushroom, and moringa leaf. Food Sci Nutr 5:911–920
320. Francisco CR, Heleno SA, Fernandes IP, Barreira JC, Calhelha RC et al (2018) Functionalization of yogurts with *Agaricus bisporus* extracts encapsulated in spray-dried maltodextrin crosslinked with citric acid. Food Chem 15:845–853
321. Glamočlija J, Soković M (2017) Fungi a source with huge potential for "mushroom pharmaceuticals". Lek Sirov 37:50–56
322. Gregori A (2014) Cordycepin production by *Cordyceps militaris* cultivation on spent brewery grains. ABS 57:45–52
323. Hassan RA, Shafi ME, Attia KM, Assar MH (2020) Influence of oyster mushroom waste on growth performance, immunity and intestinal morphology compared with antibiotics in broiler chickens. Front Vet Sci 7:33
324. Heleno SA, Rudke AR, Calhelha RC, Carocho M, Barros L et al (2017) Development of dairy beverages functionalized with pure ergosterol and mycosterol extracts: an alternative to phytosterol-based beverages. Food Funct 8:103
325. Hyde KD, Bahkali AH, Moslem MA (2010) Fungi.-.an unusual source for cosmetics. Fungal Divers 43:1–9
326. Jakopovich I (2011) New dietary supplements from medicinal mushrooms: Dr. Myko San-A registration report. Int J Med Mushrooms 13:307–313
327. Jayachandran M, Xiao J, Xu B (2017) A critical review on health promoting benefits of edible mushrooms through gut microbiota. Int J Mol Sci 18:1934
328. Kalač P (2016) Edible mushrooms. Academic Press, Amsterdam, Chemical composition and nutritional value
329. Khan SH, Mukhtar N, Iqbal J (2019) Role of mushroom as dietary supplement on performance of poultry. J Diet Suppl 16:611–624
330. Kües U, Khonsuntia W, Subba S, Dörnte B (2018) Volatiles in communication of Agaricomycetes. In: Anke T, Schüffler A (eds) Physiology and genetics, The Mycota (A comprehensive Treatise on Fungi as Experimental Systems for Basic and Applied Research), vol 15. Springer, Cham, pp 149–212
331. Li TH, Che PF, Zhang CR, Zhang B, Ali A et al (2020) Recycling of spent mushroom substrate: utilization as feed material for the larvae of the yellow mealworm *Tenebrio molitor* (Coleoptera: Tenebrionidae). PLoS One 15:e0237259
332. Liu Y, Wang J, Wang W, Zhang H, Zhang X et al (2015) The chemical constituents and pharmacological actions of *Cordyceps sinensis*. Evid Based Complement Alternat Med 2015: 575063
333. Lu X, Brennan MA, Serventi L, Liu J, Guan W et al (2018) Addition of mushroom powder to pasta enhances the antioxidant content and modulates the predictive glycaemic response of pasta. Food Chem 264:199–209

334. Mehrotra A, Calvo MS, Beelman RB, Levy E, Siuty J et al (2014) Bioavailability of vitamin D_2 from enriched mushrooms in prediabetic adults: a randomized controlled trial. Eur J Clin Nutr 68:1154–1160
335. Niego AG, Rapior S, Thongklang N, Raspé O, Jaidee W et al (2021) Macrofungi as a nutraceutical source: Promising bioactive compounds and market value. J Fungi 7:397
336. Nowak R, Nowacka-Jechalke N, Juda M, Malm A (2017) The preliminary study of prebiotic potential of Polish wild mushroom polysaccharides: the stimulation effect on *Lactobacillus* strains growth. Eur J Nutr 57:1511–1521
337. Ogbe AO, Mgbojikwe LO, Owoade AA, Atawodi SE, Abdu PA (2008) The effect of a wild mushroom (*Ganoderma lucidum*) supplementation of feed on the immune response of pullet chickens to infectious bursal disease vaccine. Electron J Environ Agric Food Chem 7:2844–2855
338. Paul N, Slathia PS, Vaid A, Kumar R (2018) Traditional knowledge of Gucchi, *Morchella esculenta* (Ascomycetes), in Doda district, Jammu and Kashmir. India Int J Med Mushrooms 20:445–450
339. Prasad S, Rathore H, Sharma S, Yadav AS (2015) Medicinal mushrooms as a source of novel functional food. Int J Food Sci Nutr Diet 04:221–225
340. Rahi DK, Rahi S, Chaudhary E (2021) White-rot fungi in food and pharmaceutical industries. In: Sridhar KR, Deshmukh SK (eds) Advances in Macrofungi: Industrial Avenues and Prospects. CRC Press, Boca Raton, pp 175–206
341. Rapior S, Mauruc MJ, Guinberteau J, Masson CL, Bessière JM (2000) The volatile composition of *Gyrophragmium dunalii*. Mycologia 92:1043–1046
342. Razif MF, Shin-Yee, Fung SY (2021) Cosmeceuticals from mushrooms. In: Sridhar KR, Deshmukh SK (eds) Advances in macrofungi: industrial avenues and prospects. CRC Press, Boca Raton, pp 230–254
343. Reis FS, Martins A, Vasconcelos MH, Morales P, Ferreira IC (2017) Functional foods based on extracts or compounds derived from mushrooms. Trends Food Sci Technol 66:48–62
344. Sękara A, Kalisz A, Grabowska A, Siwulski M (2015) *Auricularia* spp.-mushrooms as novel food and therapeutic agents-a review. Sydowia 67:1–10
345. Singdevsachan SK, Auroshree P, Mishra J, Baliyarsingh B, Tayung K et al (2016) Mushroom polysaccharides as potential prebiotics with their antitumor and immunomodulating properties: a review. Bioact Carbohydr Dietary Fibre 7:1–14
346. Singh J, Sindhu SC, Sindhu A, Yadav A (2016) Development and evaluation of value added biscuits from dehydrated Shiitake (*Lentinula edodes*) mushroom. Int J Curr Res 8:27155–27159
347. Sknepnek A, Pantić M, Matijašević D, Miletić D, Lević S et al (2018) Novel kombucha beverage from Lingzhi or Reishi medicinal mushroom, *Ganoderma lucidum*, with antibacterial and antioxidant effects. Int J Med Mushrooms 20:243–258
348. Taofiq O, Martins A, Barreiro MF, Ferreira IC (2016) Anti-inflammatory potential of mushroom extracts and isolated metabolites. Trends Food Sci Technol 50:193–210
349. Thomrongsuwannakij T, Charoenvisal N, Chansiripornchai N (2021) Comparison of two attenuated infectious bursal disease vaccine strains focused on safety and antibody response in commercial broilers. Vet World 14:70–77
350. Vargas-Sánchez RD, Torrescano-Urrutia GR, Ibarra-Arias FJ, Portillo-Loera JJ, Ríos-Rincón FG et al (2018) Effect of dietary supplementation with *Pleurotus ostreatus* on growth performance and meat quality of Japanese quail. Livest Sci 207:117–125
351. Wu Y, Choi MH, Li J, Yang H, Shin HJ (2016) Mushroom cosmetics: the present and future. Cosmetics 3:22
352. Ying M, Yu Q, Zheng B, Wang H, Wang J et al (2020) Cultured *Cordyceps sinensis* polysaccharides modulate intestinal mucosal immunity and gut microbiota in cyclophosphamide-treated mice. Carbohydr Polym 235:115957
353. Yuan B, Zhao L, Yang W, McClements DJ, Hu Q (2017) Enrichment of bread with nutraceutical-rich mushrooms: impact of *Auricularia auricula* (mushroom) flour upon quality attributes of wheat dough and bread. J Food Sci 82:2041–2050

Fungal Enzyme-Based Nutraceutical Oligosaccharides

12

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Abstract

Prebiotic oligosaccharides are receiving immense attention due to their nutraceutical and therapeutic potential associated with the food and pharmaceutical industry. Owing to increasing demand for prebiotic oligosaccharides, various strategies of their production are being pursued. Fungal enzymes, mainly fructosyltransferase, inulinase, mannanase, cellulases, galactosidase, and xylanase, are predominantly involved in the synthesis of various oligosaccharides such as fructooligosaccharides (FOS), inulooligosaccharides (IOS), mannooligosaccharides (MOS), cellooligosaccharides (COS), galactooligosaccharides (GOS), and xylooligosaccharides (XOS) through biotransformation of their respective precursor raw sugars. This chapter highlights modern approaches and production strategies of nutritional oligosaccharides using fungal enzymes and their nutritional values.

Keywords

Oligosaccharides · Prebiotics · Fructooligosaccharides · Inulooligosaccharides · Mannooligosaccharides · Cellooligosaccharides · Galactooligosaccharides · Xylooligosaccharides

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12.1 Introduction

Nutritional dependencies of the modern lifestyle are major socioeconomic concerns in our day-to-day life. Incorporation of functional food ingredients as a part of daily diet may play crucial role in the well-being. Functional foods can be a better preventive measure for mitigating chronic health disorders, such as irritable bowel disease (IBD), inflammatory disorders, indigestion, colon cancer, gut epithelial dysfunction, etc. [1]. Prebiotic dietary fibers are selectively utilized by probiotic gut microflora which provide important health benefits to the host [2]. In a first, a study evidenced lactulose as a bifidogenic factor [3]. After several years, some non-digestible oligosaccharides were found as promising bifidogenic components [4, 5]. The term "prebiotic" was coined by Gibson and Roberfroid, and they defined prebiotics as non-digestible food components that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving the host health [6].

Due to upsurge in their consumption all over the globe, the prebiotic market in 2015 was estimated to be $200 million with a growth rate of 15% per year and is expected to reach $8.5 billion by the year 2024 [7]. Nutraceutical oligosaccharides are considered as non-digestible carbohydrates that help attain balanced microbial composition in the host gut and are responsible for a number of health benefits such as reduced risk of colon cancer, improved mineral absorption, antioxidant activities, inhibition in pathogenic microbes in the gut, generation of immunomodulatory compounds, reduction of neoplastic lesions and cancer risk, improvement of bowel movement, stimulation of immune system, improvement of gastrointestinal and urinary tract health, anti-inflammatory effects, reduction of blood pressure, maintenance of vision, antibacterial and antiviral activities, and reduction of osteoporosis and lipid levels [8, 9]. They also help in improving gut barrier function and gut-brain signaling in depressive disorders [10].

Among the functional food components, prebiotics and probiotics constitute a focus on contemporary advancements in human nutrition. The market of prebiotics in India is still emerging. A few fructooligosaccharide-based products are available in Indian market, and these continue to draw more attention by the health-conscious individual. All prebiotics are considered in the group of dietary fibers, but some dietary fibers do not exhibit prebiotic activity. Major prebiotics are fructooligosaccharides (FOS), inulin and inulooligosaccharides (IOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), mannooligosaccharides (MOS), and cellooligosaccharides (COS). In addition to these, maltooligosaccharides (MaOS), isomalto-oligosaccharides (IMaOS), human milk oligosaccharides (HMOS), pectin-derived oligosaccharides (POS), agarooligosaccharides (AOS), lactulose, and lactosucrose are also being evaluated for the purpose (Table 12.1). Among these, inulin, IOS, FOS, and GOS are the most popular prebiotics and are commonly used in food and feed, confectionary, and animal feed industries [21].

Table 12.1 Natural sources of oligosaccharides

Oligosaccharides	Composition	Sources	References
Fructooligosaccharides and inulooligosaccharides	G-(F)n β (2–1) β (2–6)	Asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat, honey, banana, barley, tomato, and rye are special sources of fructooligosaccharides	[8, 11–13]
Mannooligosaccharides	G-(M)n α (1–4) β (1–4)	Yeast cell wall Locust bean gum, guar gum	[14, 15]
Xylooligosaccharides	(X)n β (1–4)	Bamboo shoots, husks, straws, and corncobs	[16, 17]
Galactooligosaccharides	G-(Ga)n β (1–3) β (1–4)	Milk, legumes, and sugar beet root	[18, 19]
Pectin	(GaA)n α (1–4)	Citrus peel, apple pomace, sugar beet	[20]

G glucose, *F* fructose, *Ga* galactose, *X* xylose, *M* mannose, *A* arabinose

12.2 Production of Prebiotic Oligosaccharides

Although prebiotic oligosaccharides are found in various natural sources (Table 12.1), the quantity and composition vary with respect to environmental conditions. The production of oligosaccharides may be done either by chemical synthesis by transglycosylation or by biocatalysis of raw sugars using various enzymes. Chemical synthesis of oligosaccharides may be attained by thioglycoside glycosylation, ortho-trichloroacetimidate method, orthoester method, condensation of tritylcyanothylidene, modified method of Koenigs-Knorr, etc. Chemical methods of oligosaccharide synthesis have disadvantages due to the uncontrolled stereochemistry and non-specificity [22].

Due to these limitations of chemical methods, oligosaccharide generation involving enzymatic bioprocesses is a very promising alternative. Several fungal enzymes such as fructosyltransferase, inulinase, mannanase, xylanase, β-galactosidase, and cellulase are being utilized for the generation of FOS, IOS, MOS, XOS, GOS, and COS, respectively (Fig. 12.1). Most of the prebiotic oligosaccharides are generated using enzymes belonging to three kinds of enzymes, viz., glycoside hydrolase (GH), glycosyltransferase (GT), and transglycosylase (TG) [23].

GH enzymes are involved in the hydrolysis of acetal linkages between two carbohydrates or between a carbohydrate and a non-carbohydrate moiety. GT enzymes catalyze the transfer of glycosidic moiety from activated donor to acceptor residue. GTs are sub-classified as inverting or retaining on the basis of the stereochemistry of the glycosidic bond (α/β) in reaction product is maintained or altered. TGs are involved in similar catalytic reaction as the GHs, but the hydrolase versus transferase ratio differs in different TGs. The mechanism and biological role of TGs is still undistinguishable [24]. The production of different types of oligosaccharides

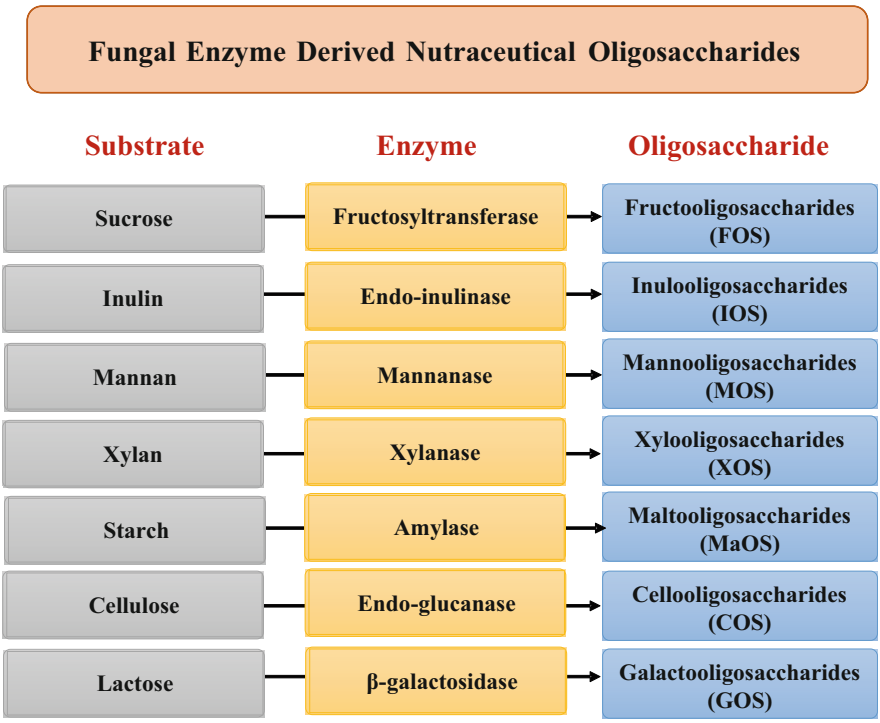

Fig. 12.1 Types of nutraceutical oligosaccharides and fungal enzymes involved in their generation

is commercially achieved using several enzymes based on their type of glycosidic linkage formation in the product oligosaccharide. The prebiotic and other nutritional properties or oligosaccharides depend upon the three-dimensional stereochemistry of the glycosidic bond, which could not be controlled by chemical catalysis. Enzymatic synthesis of oligosaccharides displays some properties such as selectivity, specificity, and energy minimized catalysis with high turnover number [24]. Furthermore, several enzyme engineering approaches such as immobilization, genetic engineering, codon optimization, and mutagenesis have also been regularly utilized to improve catalytic properties of enzymes.

Oligosaccharides have been marketed since the 1980s as low-calorie agents and recently have gained interest in the pharmaceutical and food industry as functional sweeteners and prebiotic enriching population of *Bifidobacteria*. Currently, they have an approximated value of $ 200 per kg, and recently, inulin has been proposed as a feedstock for production of oligosaccharides through selective hydrolysis by action of endo-inulinase [25]. The influences of probiotic or pre-diet on the zebrafish gut-brain axis have reported. Zebrafish (*Danio rerio*) fed with probiotic were found with increased levels of serotonin and brain-derived neurotrophic factor [26]. *Lactobacillus rhamnosus* IMC 501® (a probiotic strain) showed health-promoting properties such as decreased DNA damage, less oxidative stress, and increased

Table 12.2 Commercial oligosaccharides and their sources

Oligosaccharide	Trade name	Source/company
FOS	Actilight®	Baghin-Meji industries, Paris, France
	Meioligo®	Meiji Seika Kaisha, Tokyo, Japan
	NutraFlora®	GTC Nutrition, Golden Colorado, US
Inulin	GlaxoSmithkline, Philadelphia	FiberChoice
MOS	Bio-MOS^{R}	Alltech, US
	ActiveMOS^{R}	Orffa, the Netherlands
	AgriMOS	Lallemand Inc., Canada
GOS	Oligomate ®	Yakult Pharmaceutical Industry Co., Ltd. (Japan)
	Cup Oligo	Nissin Sugar Co., Ltd. (Japan)
	Vivinal® GOS	Friesland Campinas (the Netherlands)
XOS	PreneXOS™	Shandong Longlive Bio-Tech Co., Ltd., China
	XOS	Van Wankum Ingredients, the Netherlands
COS	D (+) Cellotriose Cellotetraose	Megazyme (Bray, Ireland)
MaOS		Sigma-Aldrich (St. Louis, MO)

immune response including hepatic stress tolerance in zebrafish [27]. Some of the commercially available oligosaccharides are listed in Table 12.2. Details of some of the nutritionally important oligosaccharides and enzymes involved in their generation are presented in the following sections.

12.3 Types of Prebiotic Oligosaccharides

12.3.1 Fructooligosaccharides (FOS) and Inulooligosaccharides (IOS)

Fructooligosaccharides (FOS) are considered to be the most important among various prebiotics due to their nutraceutical properties such as hypolipidemic (cholesterol-lowering) and enhanced calcium absorption [28, 29]. FOS consist of a series of oligosaccharides that are composed of 1-kestose (GF2), nystose (GF3), and 1F-β-fructofuranosyl nystose (GF4), in which two, three, and four fructosyl units are bound at the β-2,1 position of glucose, respectively. FOS are obtained either by extraction from various plant materials or by enzymatic synthesis from different substrates. Enzymatically, these can be obtained either from sucrose using FTase or from inulin hydrolysis by endo-inulinase [11]. Increasing demand for an alternative healthy sweetener and multifunctional fructooligosaccharides has prompted investigators to explore microorganisms for inulinase and FTase production and to develop bioprocesses for the production of high-fructose syrup [8].

Current commercial production of FOS is carried out by fructofuranosidase (FFase, EC 3.2.1.26) or fructosyltransferase (FTase, EC 2.4.1.9) using sucrose as the raw material [30, 31]. Fructosyltransferase (FTase; EC 2.4.1.9) hydrolyzes sucrose and transfers fructosyl group to an acceptor molecule to generate fructooligosaccharides (FOS) along with glucose and fructose [32]. Hidaka et al. [33] reported that *Aspergillus niger* ATCC 20611 produces β-fructofuranosidase (FopA) with high transfructosylation activity. *A. niger* ATCC 20611 is being exploited at industrial scale for FOS production in the last two decades as commercial FOS producer [34]. Recently, genetically modified *Pichia*, expressing plant fructosyltransferase, was designed for industrial production of FOS [35]. Another recent report describes neo-series FOS production using β-fructofuranosidase (FFase) derived from *Xanthophyllomyces dendrorhous*. For industrial application, the gene encoding FFase has been cloned in *Pichia*, and the expressed enzyme was immobilized on polyvinyl alcohol matrix and used for continuous generation of FOS [36]. A cold-active FTase from *Aspergillus tamarii* generated a maximum of 55% (325 g/L) FOS from sucrose under optimized biotransformation parameters [37]. Another fungus *Penicillium citrinum* produced FTase units in fermentation media containing banana peel (6.9 U/mL) and sugarcane molasses (7.3 U/mL) [38].

Prebiotic fructooligosaccharides can also be obtained by one-step hydrolysis of inulin by endo-acting inulinases (endo-inulinases). Inulin serves as a storage polysaccharide in many plants of Composite and Gramineae. It consists of β-(2–1)-D-fructosyl-fructose links terminated by a sucrose residue [8, 39, 40]. This fructan is a potential substrate for generation of high fructose syrup (HFS) and prebiotic inulooligosaccharides (IOS). Inulin is acted upon by two types of inulinases, i.e., endo-inulinase (2,1-β-D-fructanfructanohydrolase, EC 3.2.1.7) and exoinulinase (β-D-fructanfructohydrolase, EC 3.2.1.80). Endo-inulinases liberate IOS as the main product [8], while exoinulinases hydrolyze the terminal linkages to yield fructose as the main product. Pertaining to the high demand of FOS, their cost-effective production is assuming greater challenges. In this context, development of an enzyme-based process using microbial transferases and hydrolases can help achieving the target of producing FOS using cost-effective indigenous technology. High fructose syrup can be biotransformed into value added products such as ethanol and single-cell protein, while IOS are indicated in nutraceutical industry as prebiotics [41]. Bhalla et al. [42] reported FOS generation using *Saccharomyces cerevisiae* isolated from local fermented beverage called Chaang. It was selected after screening for high invertase activity. Highest yield was obtained from 250 mg sucrose concentration and 2.5 U of invertase in 1 ml reaction at pH 5.5 and 40 °C. Production of an extracellular, thermostable inulinase was carried out by *Aspergillus tubingensis* CR16 using wheat bran and corn steep liquor (CSL) under solid-state fermentation (SSF). The fungus produced 1358.6 ± 0.8 U/g inulinase after parametric optimization which was fivefold higher [43]. *Bacillus safensis* AS-08 grown on dahlia inulin produced inulinases which hydrolyzed inulin to mixture of fructooligosaccharides [44].

A. niger NK-126 showed high inulinase activity on dandelion tap root extract (52.3 U/ml) and produced a mixture of fructose and FOS from chicory inulin [45]. In

silico studies of Singh and Shukla [46] have shown that exo- and endo-inulinases from *Penicillium* sp. TN-88 have different arrangement of amino acids in the active site for recognition of substrate. Dilipkumar et al. [47] have used sugarcane press mud for the production of inulinase in solid-state fermentation (SSF). The optimized medium with sugarcane juice at 20% (v/v) and casein peptone at 2% (w/v) was found to be optimal at an initial pH 7.0 and incubation temperature 35 °C for 48 h. The produced inulin-type FOS (kestose and neokestose) and levan were characterized by Fourier transform infrared spectroscopy (FT-IR) and nuclear magnetic resonance (NMR) analysis. The study revealed that the levansucrase could form FOS from sucrose [48]. *Aspergillus fumigatus* NFCCI 2426 was found to produce 68 U/ml of FTase activity on a medium containing 20% w/v sucrose. The enzyme was partially purified (acetone precipitation followed by DEAE-Cellulose anion exchange chromatography) and immobilized in calcium alginate for continuous transfructosylation of food grade sucrose to generate FOS (GF5, GF4, GF3, GF2) and gluco-fructose (our unpublished results). The research in the field of FOS is gaining momentum among researchers because of their tremendous potential as nutraceuticals. Purama et al. [49] summarized pathways of colorectal cancer (CRC) inhibition by FOS. These oligos also play crucial role in immune health maintenance by increasing the concentration of interleukins and IgG. They inhibit cancer cell growth by activating caspase pathway for apoptotic death of CRC cells [49].

12.3.2 Galactooligosaccharides (GOS) and Other Lactose-Derived Oligosaccharides

GOS are non-digestible oligosaccharides composed of 2–8 galactose moieties linked by β (1 → 4) and β (1 → 6) bonds with a terminal glucose residue, while β (1 → 2) and β (1 → 3) glycosidic linkages may be found in some GOS [24]. GOS are usually produced by the transgalactosylation catalyzed by β-galactosidase (βG; EC 3.2.1.23) using lactose-rich solutions. The βG catalyzes transgalactosylation in a similar way to the fructosyltransferase in a kinetically controlled manner [50]. The transgalactosylation reaction proceeds through the release of galactose from lactose followed by its transfer to another lactose molecule to generate trisaccharide (GOS 3). Subsequent formation of GOS 4, 5, and 6 occurs with GOS 3, 4, and 5 as an acceptor molecule. βGs are obtained from a variety of microorganisms mainly from *Aspergillus* spp. (*A. niger* and *A. oryzae*), yeasts (*Kluyveromyces* spp.), and bacteria (*Bacillus circulans*) as prominent producers.

GOS are frequently used as functional food ingredient in dairy products, bakery, and beverages. Recently, a novel β-galactosidase from the fungus *Thermothielavioides terrestris* was heterologously expressed for the production of GOS. The recombinant enzyme resulted in 19.4% and 14.8% GOS yield from lactose solutions and acid whey, respectively [51]. On the other hand, bacterial galactosidase (GH family 42) from *Pantoea anthophila* resulted in 40% GOS yield corresponding to 86% lactose conversion from 400 g/L lactose. The lactose hydrolysate was composed of 14% GOS, 5.7% 6-galactobiose, 20.2% allolactose, 46.7%

mixed monosugars (glucose and galactose), and 13.3% residual lactose [52]. Another acidic β-galactosidase from *A. oryzae* (ENZECO), having optimum pH 4.5, produced a maximum of 26.73% GOS (DP 3 and 4) from lactose solution [53]. β-Galactosidase from *A. oryzae* (≥8 IU mg^{-1}; Sigma Chemicals Co, USA) was utilized to generate GOS from whey powder substrate. Maximum yield of 62 g L^{-1} of GOS was obtained from 40% sweet whey powder bioconversion. Also, the immobilization of this enzyme on a synthetic methacrylic immobilization carrier (Lifetech ECR8409) resulted in 2.5-fold enhanced GOS productivity and recyclability over 4 cycles [54]. Commercial β-galactosidase preparations sourced from *Aspergillus aculeatus*, *A. oryzae*, *A. niger*, *B. circulans*, and *Kluyveromyces lactis* have been employed for GOS production from lactose and lactulose solutions. Among all the tested enzyme preparations, fungal lactase sourced from *A. oryzae* yielded maximum GOS (0.29 g g^{-1} from lactose and 0.38 g g^{-1} from lactulose) [55].

12.3.3 Cellooligosaccharides (COS)

Cellulose is the most abundant structural polysaccharide on the earth. Cellulose (poly β-1,4-glucopyranose) is composed of closely knit linear chains of anhydroglucose monomers linked by β-1,4-glycosidic linkages at the C1 and C4 positions. Cellooligosaccharides (COS) display the same chemical structure as cellulose but have DP in the range of 2–6. Although cellulose is insoluble in water, due to smaller DP, COS are fairly soluble in water at room temperature. COS are generated by depolymerization and glycosylation of cellulose polymer. Glycosidic linkages other than β-1,4 glycosidic bonds such as α-1,4, α/β-1,3, α/β-1,6, and α/β-1,2-glycosidic linkages may be found in COS [56]. COS are mainly derived by controlled de-polymerization from plant cellulose. COS can be obtained by acid or enzymatic hydrolysis of plant cellulose. Partial hydrolysis of cellulosic biomass is based on the protonation of glycosidic bond by acidic or enzymatic catalysis. Among the entire COS synthesis approaches, enzymatic de-polymerization of cellulose is the most promising, eco-friendly, and economical approach.

COS synthesis by enzymatic catalysis is generally done either from sucrose and glucose biotransformation employing a catalytic cascade involving three glycoside phosphorylases, namely, sucrose phosphorylase (ScP; GH 13 family), cellobiose phosphorylase (CbP; GH 94 family), and cellodextrin phosphorylase (CdP; GH 94 family) [57–59] or by hydrolyzing cellulose using cellulases [60]. Cellulase consortium predominantly contains three enzymes: (1) endo-1,4-glucanase (EG) (EC 3.2.1.4), responsible for the endo-hydrolysis of cellulose polymer at internal amorphous sites generating cello-oligomers (high DP); (2) cellobiohydrolases (CBH) (EC 3.2.1.91) or exo-1,4-glucanases, catalyze the exo-hydrolysis of crystalline cellulose at the reducing end to generate cellobiose and short-chain COS; and (3) β-glucosidases (βG) (E.C. 3.2.1.21), which hydrolyze the produced cellobiose to glucose monomers [61]. A controlled enzymatic hydrolysis of cellulose using different commercial cellulase combinations (CBH from *Trichoderma longibrachiatum* and EG from *Thermothelomyces thermophila*) has

been demonstrated to generate cellobiose. Cellulase combination (CBHI/EG5) in a ratio of 80:20 led to the generation of optimal yields (49.7% w/w) of cellobiose with the cellobiose/glucose ratio of 9.4 [60]. Details on the production of COS from lignocellulosic biomass and their prebiotic applications have been compiled by Avila et al. [62]. Production of cellobiose from organosolv-pretreated birch lignocellulose hydrolysis using an optimum combination of cellulases (GH family 5 endoglucanase sourced from *Talaromyces emersonii*, GH family 6 cellobiohydrolase from *Podospora anserina*, GH family 7 cellobiohydrolase, and GH family 7 endoglucanase from *T. thermophila* with an accessory enzyme lytic polysaccharide monooxygenase from *T. thermophila*) led to production of cellobiose (22.3%) [63]. Zhou et al. [64] demonstrated an effective strategy to produce purified COS by simultaneous production of sugar monoesters to remove monosugars. This strategy resulted in an improvement in COS production from 33.3 to 74.3%. On the other hand, controlled synthesis of ammonia- pretreated wheat straw depolymerization had been achieved using some of the adsorbed activities of cellulase cocktail obtained from *A. niger*. Cellulase adsorption (majorly exocellulase) on cellulosic biomass favored cellobiose synthesis, while the unadsorbed liquid fraction predominantly produced other COS [65]. Selective removal of β-glucosidase activity from cellulase pool resulted in a 36% increased COS production from corncob residue with a yield of 51.78% than single-stage hydrolysis [66].

12.3.4 Mannooligosaccharides (MOS)

Mannans, consisting of D-mannose linked by β-1,4 mannosidic linkages, are found in coffee beans, locust bean gum (LBG), guar gum (GG), palm kernel cake (PKC), konjac gum (KG), ivory nuts, sugar beets, soybeans, etc. Mannans are of four types based on their glycosidic linkage and branching patterns [67, 68]. Oligomers of mannose, mannooligosaccharides (MOS), are classified into alpha or beta types. The α-MOS are mainly obtained by hydrolysis of yeast cell wall, while β-MOS are mainly produced by hydrolysis of plant mannans by chemical (acid/alkaline), physical (ultrasonic), or enzymatic (mannanase) means [69].

β-MOS are generated using enzyme consortia consisting of β-1, 4-mannanase (EC 3.2.1.78), β-mannosidase (EC3.2.1.25), α-galactosidase (EC 3.2.1.22), and β-glucosidase (EC 3.2.1.21) [15, 70]. According to the sequence similarity database of catalytic sequences, they are grouped into glycoside hydrolase (GH) families – 5, 26, 113, and 134 (http://www.cazy.org/) [71]. Several reports describe the production of MOS from agro-industrial wastes using microbial β-mannanases [72]. Among these, fungal mannanases are for high-yield generation of MOS. Many fungal sources have been reported to produce β-mannanases that belong to family GH-5 and GH-26. Mannanases from *Yunnania penicillata*, *Aspergillus nidulans*, *A. niger*, *A. oryzae*, and *Rhizomucor miehei* have been reported to produce MOS [67, 71, 73–76]. Li and co-workers [77] engineered *R. miehei* β-mannanase and heterologously expressed in *Pichia pastoris*. This engineered mannanase

produced 34.8 g MOS per 100 g dry palm kernel cake with 80.6% hydrolysis yield. Additionally, *Penicillium oxalicum* β-mannanase was employed to generate MOS from copra meal and coffee rests. Their hydrolyzed products were composed of mannose, M2, M4, and M6. Recombinant mannanase (1625 U/mL) was utilized for the production of MOS from copra meal and palm kernel meal. Copra meal generated M2 as major product, while mannose and M2 were the major products with smaller amounts of M3 in case of palm kernel meal [78]. β-Mannanase from *Talaromyces trachyspermus* generated mainly mannose and M2 from coffee waste and M2, M3, and M4 from locust bean gum [79].

Among microbial sources, mannanases (mainly GH5 and GH26) from filamentous fungus *A. niger* are the most widely studied for MOS production. Commercial production of MOS requires robust enzyme which is suitable for industrial applications [76]. A codon-optimized mannanase (AnMan26) from *A. niger* was expressed in *Pichia pastoris*, and titers to the tune of 22,100 U mL^{-1} were obtained in a 5-L fermenter. It had maximum specific activity toward locust bean gum and produced mannooligosaccharides from locust bean galactomannan (LBG). Moreover, it also resulted in the production of high DP MOS (1.8×10^3 Da) from partial hydrolysis of fenugreek gum [76]. β-Mannanases from *A. oryzae*, *A. quadrilineatus*, *Aspergillus terreus*, and thermophilic *Malbranchea cinnamomea* were characterized and used to produce MOS from LBG, guar gum, palm kernel cake, and copra meal [67, 80–82].

12.3.5 Xylooligosaccharides (XOS)

Xylan is a low molecular weight (DP 80–200) plant polysaccharide mainly found in the form of cell wall hemicellulose. Xylans are branched polymers of (1 → 4) linked β-D-xylopyranosyl backbones. Branched chains may be substituted with ferulic acid, acetyl, 4-O-methyl glucuronic acid, p-coumaric acid, or an arabinose side group [8, 83, 84]. Plant hemicelluloses having xylose and arabinose with traces of uronic acid (glucuronic acid and 4-O-methyl derivative) are termed as arabinoxylans, while glucose linked xylan are termed as gluco-xylans [85, 86]. Xylooligosaccharides are (1 → 4) linked β-D-xylopyranose oligomers (DP 2–7) with varying properties such as degree of polymerization and structural properties depending upon the raw source used. Xylan may be extracted from plant cell wall using water [87], acid treatment [88], alkali [89, 90], dimethyl sulfoxide (DMSO) [91, 92], or hot and cold water under pressure [93]. Xylooligosaccharides can be produced by enzymatic hydrolysis of β-1,4-xylosidic bonds of xylan by endo-1,4-β-D-xylanases (EC 3.2.1.8). Endo-xylanases mainly belong to glycoside hydrolase (GH) families 10 and 11, while some xylanases also belong to other GH families (5, 7, 8, 16, 26, 30, 43, 52, and 62) [94]. As an emerging prebiotic, XOS exhibit health benefitting properties such as bifidogenic potential [95] and increased calcium absorptivity, minimize colon cancer risk, and confer immune-regulatory properties [96]. They also display some other medicinal properties such as antioxidant, anti-allergic, anti-inflammatory, and cytotoxic properties [94, 97]. Several fungal strains

such as *Paecilomyces variotii*, *A. terreus*, *A. fumigatus*, *Penicillium glabrum*, *Sorangium cellulosum*, *Thermomyces lanuginosus*, and *M. cinnamomea* have been reported to produce high titers of xylanases [98–103]. Xylanase secreted by *P. variotii* resulted in the generation of XOS composed of xylobiose (X2 14%), xylotriose (X3 27%), and xylotetrose (X4 23%), together with a small amount of xylopentaose (X5 18%) and xylohexose (X6 13%) and xylose (X1 0.8%) in 0.5 h hydrolysis of 1% w/v beechwood at 55 °C [94].

Brenelli et al. [104] described an interesting approach using slight acetylation followed by hydrothermal pretreatment for improved XOS production from sugarcane straw (SS) catalyzed by *Aspergillus nidulans* xylanase (GH 10). The pretreatment strategy promoted 81.5% hemicellulose solubilization and resulted in XOS (X2, X3) yield up to 9.8%. Xylanase from *T. lanuginosus* produced XOS composed of X2 (66.46%), X3 (25.10%), and small amount of xylose (4.97%) from beechwood xylan [105]. Commercial xylanase from *T. longibrachiatum* produced 44.43% XOS from Brewers' spent grain over 12 h hydrolysis [106]. Endoxylanase from *Streptomyces thermovulgaris* was utilized to produce XOS (10.66%) from pretreated corn cobs [107]. Other raw sources such as coconut husk, finger millet seed coat, rice bran, sugarcane bagasse, wheat straw, etc. have been utilized for XOS production using bacterial and fungal xylanases [108–111]. Corn cob xylan (2% w/v) treated with partially purified *T. lanuginosus* xylanase for 8 h at 45 °C yielded 6.9 mg/ml of XOS (X2, X3) [112].

12.3.6 Maltooligosaccharides (MaOS)

Maltooligosaccharides (MaOS) are composed of 2–10 units of α-1,4-linked glucopyranose monomers. MaOS are generally produced from starch by the catalytic action of α-amylase (EC 3.2.1.1) [113]. MaOS generating amylases have been described from several bacterial species [114] and also from a few fungal species such as *A. niger* and *A. nidulans* [56, 115]. Kazim et al. [115] characterized the MaOS generating ability of an amylase (AmyG) sourced from *A. nidulans*. The AmyG generated DP3 to DP6 MaOS from starch hydrolysis. In another study, high concentration (1 μM) of *A. niger* amylase has been applied to hydrolyze corn starch, potato starch, and wheat starch for 12 h to generate MaOS (DP 1–3). The enzyme treatment produced a maximum of 16 mg/mL MaOS from potato starch while 14 mg mL^{-1} from corn and wheat starch with trace amounts of DP 4 MaOS [56].

On the other hand, some reports of MaOS generating amylosucrase (EC 2.4.1.4, ASase) are also available. This enzyme exhibits glucosyltransferase activity and catalyzes MaOS synthesis using sucrose as the substrate. A comparative study on two-step and one-step production strategies of MaOS (DP 3–6) using bacterial amylases has been carried out by Zhu et al. [113].

12.4 Nutritional Aspects of Prebiotic Oligosaccharides

Pro-health properties of oligosaccharides made them a very important ingredient among functional foods. Most of the oligosaccharides are known to exhibit multifarious nutritional benefits through modulation of the gut microbiota towards healthy-gut environment [116, 117] and imparting antioxidant, immune-booster effects, and high mineral (especially calcium and magnesium) absorptivity through gut epithelium [118]. Moreover, they also help in reduction of some metabolic disorders such as cardiovascular diseases (CVD) [119], inflammable Bowel's disease (IBD), major depressive disorder (MDD) [120], and obesity [121, 122]. These properties have highlighted oligosaccharides as an important nutraceutical additive in food and feed industry, juice and beverage industry, cosmetic applications, medicinal applications, animal feed and livestock applications, etc. Recently, fructooligosaccharides and inulin (dried Jerusalem artichoke tubers) have been shown to produce positive effect on the pork quality and fatty acid profile. Improved antioxidant status, water-holding capacity, and a reduced shear force was observed. Furthermore, prebiotic addition in the pig diet improved the quality and shelf-life of the pork [122]. Prebiotic-enriched diet also contributed to a better weight gain and inhibition in diarrhea in piglets [123]. Inulin oligosaccharides may reduce the activity and expression of fat-generating enzymes in liver and inhibit the fatty acid synthesis and, therefore, can be used in fatteners [124]. XOS catalyzed from *P. variotii* xylanase exhibited potent antioxidant activity toward DPPH free radicals [94]. Gao et al. [118] determined the effect of GOS on the colonic mucosa of LPS-challenged piglets. GOS consumption resulted in reduction of reactive oxygen species (ROS) and malondialdehyde (MDA) and improvement in total antioxidant capacity in the injured piglets. Also, enhanced production of total short-chain fatty acids (SCFAs) was observed in LPS-challenged suckling piglets. Additionally, GOS significantly played role in immune modulation via reduced production of inflammatory molecules, interleukin 1β (IL-1β), interleukin 6 (IL-6), myeloid differentiation primary response 88 (MyD88), tumor necrosis factor-α (TNF-α), and cluster of differentiation 14 (CD14) in injured piglets. Recently, GOS have also been demonstrated to be beneficial in modulating gut microbiome of lactose-intolerant patients [116]. Probiotic growth promotion, anticancer, and antioxidant potential of MOS produced using fungal mannanase has been investigated by Jana et al. [125]. Another study described the evaluation of MOS-enriched diet for 60 days over the white leg *Litopenaeus vannamei* shrimp. MOS diet amended the productivity by 30% improved survival of shrimps. Next-generation sequencing suggested that MOS improved the *Actinobacteria* (28%) as predominant gut microbiota and inhibition in opportunistic pathogens such as *Bergeyella*, *Vibrio*, *Aeromonas*, and *Shewanella* [126]. Prebiotic potential of birch- and spruce-derived COS against *Lactobacillus* and *Bifidobacterium* has been demonstrated. Growth rate and cell density of probiotic strains was improved in the medium comprising COS as sole carbon source [63]. Similar to other oligosaccharides, MaOS are important functional food ingredient with low sweetness and osmolality and high water-holding capacity which may be utilized as

sucrose substitute [127–129]. They also exhibit immunomodulatory properties and participate in improved colonic microbiome with the reduction in pathogenic microbes [113, 130].

12.5 Conclusions and Future Prospects

Oligosaccharides, particularly FOS and GOS, are most explored prebiotics, while nutraceutical propertied of MOS, COS, XOS, and MaOS are being explored toward disease alleviation. Fungal enzymes (either hydrolases or transferases) are a good source for commercial preparation of these oligosaccharides from raw agro-waste sources. Production yield, biological properties, and the ease of commercialized production of nutraceutical oligosaccharides are majorly dependent on the source and catalytic properties of these fungal enzymes. Industrial process requires improved catalytic properties such as stability over a broad range of pH and temperature, high product yield, utilization of waste biomass as substrate, high shelf life, etc. Fungal enzymes are suitable with respect to these parameters; therefore, they are being a hot-spot for applied research on oligosaccharides for functional food industries.

References

1. Ibañez E, Cifuentes A (2013) Benefits of using algae as natural sources of functional ingredients. J Sci Food Agric 93:703–709
2. de Borba GD, Cinelli LP, Simas NK, Pessoa A, Sette LD (2019) Marine prebiotics: polysaccharides and oligosaccharides obtained by using microbial enzymes. Food Chem 280:175–186
3. Petuely F (1957) Bifidusflora bei flaschenkindern durch bifidogene substanzen (Bifidusfaktor). Z Kinderheilkunde 79:174–179
4. Yazawa K, Tamura Z (1982) Search for sugar sources for selective increase of bifidobacteria. Bifidobacteria Microflora 1:34–44
5. Yazawa K, Imai K, Tamura Z (1978) Oligosaccharides and polysaccharides specifically utilizable by bifidobacteria. Chem Pharm Bull 26:3306–3311
6. Gibson GR, Roberfroid M (1995) Dietary modulation of the human colonic microbiota -introducing the concept of prebiotics. J Nutr 125:1401–1412
7. Martins GN, Ureta MM, Tymczyszyn EE, Castilho PC, Gomez-Zavaglia A (2019) Technological aspects of the production of fructo and galacto-oligosaccharides. Enzymatic synthesis and hydrolysis Front Nutr 6:78
8. Kango N, Jain SC (2011) Production and properties of microbial inulinases: recent advances. Food Biotechnol 25:165–212
9. Singh SP, Jadaun JS, Narnoliya LK, Pandey A (2017) Prebiotic oligosaccharides: special focus on fructooligosaccharides, its biosynthesis and bioactivity. Appl Biochem Biotechnol 183:613–635
10. Choukade R, Kango N (2021) Production, properties and applications of fructosyltransferase: a current appraisal. Crit Rev Biotechnol 41:1178–1193
11. Choukade R, Kango N (2020) Applications of fungal inulinases. In: Zaragoza O, Casadevall A (eds) Encyclopedia of mycology, reference module in life sciences. Elsevier, pp 337–347

12. Itaya NM, Asega AF, Carvalho MAM, Rita de Cássia L (2007) Hydrolase and fructosyltransferase activities implicated in the accumulation of different chain size fructans in three Asteraceae species. Plant Physiol Biochem 45:647–656
13. Sangeetha PT, Ramesh MN, Prapulla SG (2005) Recent trends in the microbial production, analysis and application of fructooligosaccharides. Trends Food Sci Technol 16:442–457
14. Garcia Diaz T, Ferriani Branco A, Jacovaci FA, Cabreira Jobim C, Pratti Daniel JL, Iank Bueno AV, Ribeiro MG (2018) Use of live yeast and mannoligosaccharides in grain-based diets for cattle: ruminal parameters, nutrient digestibility, and inflammatory response. PLoS One 13:e0207127
15. Jana UK, Kango N, Pletschke B (2021) Hemicellulose-derived oligosaccharides: emerging prebiotics in disease alleviation. Front Nutr 8:670817
16. Moure A, Gullón P, Domínguez H, Parajó JC (2006) Advances in the manufacture, purification and applications of xylooligosaccharides as food additives and nutraceuticals. Process Biochem 41:1913–1923
17. Voragen AG (1998) Technological aspects of functional food-related carbohydrates. Trends in Food Sci Technol 9:328–335
18. Intanon M, Arreola SL, Pham NH, Kneifel W, Haltrich D, Nguyen TH (2014) Nature and biosynthesis of galactooligosaccharides related to oligosaccharides in human breast milk. FEMS Microbiol Lett 353:89–97
19. Martínez-Villaluenga C, Frías J (2014) Production and bioactivity of oligosaccharides in plant foods. In: Moreno FJ, Sanz ML (eds) Food oligosaccharides: production, analysis and bioactivity. John Wiley & Sons, Ltd., pp 35–54
20. Holck J, Hjernø K, Lorentze A, Vigsnæs LK, Hemmingsen L, Licht TR, Mikkelsen JD, Meyer AS (2011) Tailored enzymatic production of oligosaccharides from sugar beet pectin and evidence of differential effects of a single DP chain length difference on human faecal microbiota composition after in vitro fermentation. Process Biochem 46:1039–1049
21. Vandenplas Y, Zakharova I, Dmitrieva Y (2015) Oligosaccharides in infant formula: more evidence to validate the role of prebiotics. Br J Nutr 113:1339–1344
22. Rajagopalan G, Krishnan C (2019) Functional oligosaccharides: production and action. In: Rathinam NK, Sani RK (eds) Next generation biomanufacturing technologies. ACS Publications, American Chemical Society, Washington, DC, pp 155–180
23. Benkoulouche M, Fauré R, Remaud-Siméon M, Moulis C, André I (2019) Harnessing glycoenzyme engineering for synthesis of bioactive oligosaccharides. Interface Focus 9: 20180069
24. Vera C, Illanes A, Guerrero C (2020a) Enzymatic production of prebiotic oligosaccharides. Curr Opin Food Sci 37:160–170
25. Flores-Maltos DA, Mussatto SI, Contreras-Esquivel JC, Rodríguez-Herrera R, Teixeira JA, Aguilar CN (2016) Biotechnological production and application of fructooligosaccharides. Crit Rev Biotechnol 36:259–267
26. Borrelli L, Coretti L, Dipineto L, Bovera F, Menna F, Chiariotti L, Nizza A, Lembo F, Fioretti A (2017) Insect-based diet, a promising nutritional source, modulates gut microbiota composition and SCFAs production in laying hens. Sci Rep 7:6269
27. Gioacchini G, Giorgini E, Olivotto I, Maradonna F, Merrifield DL, Carnevali O (2014) The influence of probiotics on zebrafish *Danio rerio* innate immunity and hepatic stress. Zebrafish 11:98–106
28. Dominguez AL, Rodrigues LR, Lima NM, Teixeira JA (2014) An overview of the recent developments on fructooligosaccharide production and applications. Food Bioprocess Technol 7:324–337
29. Maiorano AE, Piccoli RM, da Silva ES, Rodrigues MFDA (2008) Microbial production of fructosyltransferases for synthesis of prebiotics. Biotechol Lett 30:1867–1877
30. Ghazi I, Fernandez-Arrojo L, Garcia-Arellano H, Ferrer M, Ballesteros A, Plou FJ (2007) Purification and kinetic characterization of a fructosyltransferase from *Aspergillus aculeatus*. J Biotechnol 128:204–211

31. Kurakake M, Masumoto R, Maguma K, Kamata A, Saito E, Ukita N, Komaki T (2010) Production of fructooligosaccharides by β-fructofuranosidases from *Aspergillus oryzae* KB. J Agric Food Chem 58:488–492
32. Ganaie MA, Gupta US, Kango N (2013) Screening of biocatalysts for transformation of sucrose to fructooligosaccharides. J Mol Catal B Enzym 97:12–17
33. Hidaka H, Hirayama M, Sumi N (1988) A fructooligosaccharide-producing enzyme from *Aspergillus niger* ATCC 20611. Agric Biol Chem 52:1181–1187
34. Trollope KM, Van Wyk N, Kotjomela MA, Volschenk H (2015) Sequence and structure-based prediction of fructosyltransferase activity for functional subclassification of fungal GH32 enzymes. FEBS J 282:4782–4796
35. Hernández L, Menéndez C, Pérez ER, Martínez D, Alfonso D, Trujillo LE, Ramírez R, Sobrino A, Mazola Y, Musacchio A, Pimentel E (2018) Fructooligosaccharides production by *Schedonorus arundinaceus* sucrose:sucrose 1-fructosyltransferase constitutively expressed to high levels in *Pichia pastoris*. J Biotechnol 266:59–71
36. Míguez N, Gimeno-Pérez M, Fernández-Polo D, Cervantes FV, Ballesteros AO, Fernández-Lobato M, Ribeiro MH, Plou FJ (2018) Immobilization of the β-fructofuranosidase from *Xanthophyllomyces dendrorhous* by entrapment in polyvinyl alcohol and its application to neo-fructooligosaccharides production. Catalysts 8:201
37. Choukade R, Kango N (2019) Characterization of a mycelial fructosyltransferase from *aspergillus tamarii* NKRC 1229 for efficient synthesis of fructooligosaccharides. Food Chem 286:434–440
38. Jayalakshmi J, Sadiq AM, Sivakumar V (2021) Microbial enzymatic production of fructooligosaccharides from sucrose in agricultural harvest. Asian Jr. of Microbiol. Biotech Env Sc 23:84–88
39. Chi Z, Chi Z, Zhang T, Liu G, Yue L (2009) Inulinase-expressing microorganisms and applications of inulinases. Appl Microbiol Biotechnol 82:211–220
40. Liu GL, Chi Z, Chi ZM (2013) Molecular characterization and expression of microbial inulinase genes. Crit Rev Microbiol 39:152–165
41. Rawat HK, Soni H, Treichel H, Kango N (2017) Biotechnological potential of microbial inulinases: recent perspective. Crit Rev Food Sci Nutr 57:3818–3829
42. Bhalla TC, Thakur B, Savitri N, Thakur N (2017) Invertase of *Saccharomyces cerevisiae* SAA-612: production, characterization and application in synthesis of fructo-oligosaccharides. LWT Food Sci Technol 77:178–185
43. Trivedi S, Divecha J, Shah A (2012) Optimization of inulinase production by a newly isolated *Aspergillus tubingensis* CR16 using low cost substrates. Carbohydr Polym 90:483–490
44. Singh RS, Singh RP (2014) Response surface optimization of endoinulinase production from a cost effective substrate by *Bacillus safensis* AS-08 for hydrolysis of inulin. Biocatal Agric Biotechnol 3:365–372
45. Kango N (2008) Production of inulinase using tap roots of dandelion *(Taraxacum officinale) by Aspergillus niger*. J Food Eng 85:473–478
46. Singh PK, Shukla P (2012) Molecular modeling and docking of microbial inulinases towards perceptive enzyme–substrate interactions. Indian J Microbiol 52:373–380
47. Dilipkumar M, Rajasimman M, Rajamohan N (2014) Utilization of copra waste for the solid state fermentative production of inulinase in batch and packed bed reactors. Carbohydr Polym 102:662–668
48. Xavier JR, Ramana KV (2016) Optimization of Levan production by cold-active *Bacillus licheniformis* ANT 179 and fructooligosaccharide synthesis by its levansucrase. Appl Biochem Biotechnol 181:986–1006
49. Purama RK, Raman M, Ambalam P, Pithva S, Kothari C, Doble M (2018) Prebiotics and probiotics in altering microbiota: implications in colorectal cancer. In: Chatterjee S, Jungraithmayr W, Bagchi D (eds) Immunity and inflammation in health and disease. Academic Press, Elsevier, pp 403–413

50. Vera C, Guerrero C, Aburto C, Cordova A, Illanes A (2020b) Conventional and non-conventional applications of β-galactosidases. Biochim Biophys Acta - Proteins Proteomics 1868:140271
51. Zerva A, Limnaios A, Kritikou AS, Thomaidis NS, Taoukis P, Topakas E (2021) A novel thermophile β-galactosidase from *Thermothielavioides terrestris* producing galactooligosaccharides from acid whey. New Biotechnol 63:45–53
52. Yañez-Ñeco CV, Cervantes FV, Amaya-Delgado L, Ballesteros AO, Plou FJ, Arrizon J (2021) Synthesis of β (1 → 3) and β (1 → 6) galactooligosaccharides from lactose and whey using a recombinant β-galactosidase from *Pantoea anthophila*. Electron J Biotechnol 49:14–21
53. Cinar K, Gunes G, Gulec HA (2020) Enzymatic synthesis of prebiotic carbohydrates from lactose: kinetics and optimization of transgalactosylation activity of β-galactosidase from *Aspergillus oryzae*. J Food Process Eng 2020:e13435
54. Simović M, Milivojević A, Ćorović M, Banjanac K, Bezbradica D (2019) Whey valorization using transgalactosylation activity of immobilized β-galactosidase. Int J Food Sci Technol 54: 3074–3082
55. Guerrero C, Vera C, Conejeros R, Illanes A (2015) Transgalactosylation and hydrolytic activities of commercial preparations of β-galactosidase for the synthesis of prebiotic carbohydrates. Enzym Microb Technol 70:9–17
56. Chen P, Shrotri A, Fukuoka A (2021) Synthesis of cello-oligosaccharides by depolymerization of cellulose: a review. Appl Catal A Gen 621:118177
57. Puchart V (2015) Glycoside phosphorylases: structure, catalytic properties and biotechnological potential. Biotechnol Adv 33:261–276
58. Zhong C, Ukowitz C, Domig K, Nidetzky B (2020a) Short-chain cello-oligosaccharides: intensification and scale-up of their enzymatic production and selective growth promotion among probiotic bacteria. Agric Food Chem 68:8557–8567
59. Zhong C, Duic B, Bolivar JM, Nidetzky B (2020b) Three-enzyme phosphorylase cascade immobilized on solid support for biocatalytic synthesis of cello-oligosaccharides. ChemCatChem 12:1350–1358
60. Hrůzová K, Matsakas L, Karnaouri A, Norén F, Rova U, Christakopoulos P (2021) Valorization of outer tunic of the marine filter feeder *Ciona intestinalis* towards the production of second-generation biofuel and prebiotic oligosaccharides. Biotechnol Biofuels 14:32
61. Kango N, Jana UK, Choukade R (2019) Fungal enzymes: sources and biotechnological applications. In: Satyanarayana T, Deshmukh S, Deshpande M (eds) Advancing Frontiers in Mycology & Mycotechnology. Springer, Singapore, pp 515–538
62. Ávila PF, Silva MF, Martins M, Goldbeck R (2021) Cello-oligosaccharides production from lignocellulosic biomass and their emerging prebiotic applications. World J Microbiol Biotechnol 37:73
63. Karnaouri A, Matsakas L, Krikigianni E, Rova U, Christakopoulos P (2019) Valorization of waste forest biomass toward the production of cello-oligosaccharides with potential prebiotic activity by utilizing customized enzyme cocktails. Biotechnol Biofuels 12:1–19
64. Zhou P, Liu C, Wang W, Wang F, Nie K, Deng L (2020) The effectively simultaneous production of cello-oligosaccharide and glucose mono-decanoate from lignocellulose by enzymatic esterification. Appl Biochem Biotechnol 192:600–615
65. Birhade S, Pednekar M, Sagwal S, Odaneth A, Lali A (2017) Preparation of cellulase concoction using differential adsorption phenomenon. Prep Biochem Biotechnol 47:520–529
66. Chu Q, Li X, Xu Y, Wang Z, Huang J, Yu S, Yong Q (2014) Functional cello-oligosaccharides production from the corncob residues of xylo-oligosaccharides manufacture. Process Biochem 49:1217–1222
67. Jana UK, Suryawanshi RK, Prajapati BP, Kango N (2020) Prebiotic mannooligosaccharides: synthesis, characterization, and bioactive properties. Food Chem 342:128328
68. Singh S, Ghosh A, Goyal A (2018) Manno-oligosaccharides as prebiotic-valued products from agro-waste. In: Varjani S, Parameswaran B, Kumar S, Khare S (eds) Biosynthetic technology

and environmental challenges, Energy, environment, and sustainability. Springer, Singapore, pp 205–221
69. Dhawan S, Kaur J (2007) Microbial mannanases: An overview of production and applications. Crit Rev Biotechnol 27:197–216
70. Wang J, Zeng D, Liu G, Wang S, Yu S (2014) Truncation of a mannanase from *Trichoderma harzianum* improves its enzymatic properties and expression efficiency in *Trichoderma reesei*. J Ind Microbiol Biotechnol 41:125–133
71. Srivastava PK, Kapoor M (2017) Production, properties, and applications of endo-β-mannanases. Biotechnol Adv 35:1–19
72. Rungruangsaphakun J, Keawsompong S (2018) Optimization of hydrolysis conditions for the mannooligosaccharides copra meal hydrolysate production. 3 Biotech 8:169
73. Li YO, Komarek AR (2017) Dietary fibre basics: health, nutrition, analysis, and applications. Food Qual Saf 1:47–59
74. Von Freiesleben P, Moroz OV, Blagova E, Wiemann M, Spodsberg N, Agger JW, Davies GJ, Wilson KS, Stålbrand H, Meyer AS, Krogh KBRM (2019) Crystal structure and substrate interactions of an unusual fungal non-CBM carrying GH26 endo-β-mannanase from *Yunnania penicillata*. Sci Rep 9:2266
75. Von Freiesleben P, Spodsberg N, Blicher TH, Anderson L, Jørgensen H, Stålbrand H, Meyer AS, Krogh KBRM (2016) An *Aspergillus nidulans* GH26 endo-β-mannanase with a novel degradation pattern on highly substituted galactomannans. Enzym Microb Technol 83:68–77
76. Wang NN, Liu J, Li YX, Ma JW, Yan QJ, Jiang ZQ (2021) High-level expression of a glycoside hydrolase family 26 β-mannanase from *Aspergillus niger* in *Pichia pastoris* for production of partially hydrolysed fenugreek gum. Process Biochem 100:90–97
77. Li Y, Yi P, Liu J, Yan Q, Jiang Z (2018) High-level expression of an engineered β-mannanase (mRmMan5A) in *Pichia pastoris* for manno-oligosaccharide production using steam explosion pretreated palm kernel cake. Bioresour Technol 256:30–37
78. Harnpicharnchai P, Pinngoen W, Teanngam W, Sornlake W, Sae-Tang K, Manitchotpisit P, Tanapongpipat S (2016) Production of high activity aspergillus Niger BCC4525 β-mannanase in Pichia pastoris and its application for mannooligosaccharides production from biomass hydrolysis. Biosci Biotechnol Biochem 80:2298–2305
79. Suzuki K, Michikawa M, Sato H, Yuki M, Kamino K, Ogasawara W, Fushinobu S, Kaneko S (2018) Purification, cloning, functional expression, structure, and characterization of a thermostable β-mannanase from *Talaromyces trachyspermus* B168 and its efficiency in production of mannooligosaccharides from coffee wastes. J Appl Glycosci 65:13–21
80. Ahirwar S, Soni H, Rawat HK, Ganaie A, Pranaw K, Kango N (2016) Production optimization and functional characterization of thermostable β-mannanase from *Malbranchea cinnamomea* NFCCI 3724 and its applicability in mannotetraose (M4) generation. J Taiwan Inst Chem Eng 63:344–353
81. Soni H, Rawat HK, Pletschke BI, Kango N (2016) Purification and characterization of β-mannanase from *Aspergillus terreus* and its applicability in depolymerization of mannans and saccharification of lignocellulosic biomass. 3 Biotech 6:136
82. Suryawanshi RK, Kango N (2021) Production of mannooligosaccharides from various mannans and evaluation of their prebiotic potential. Food Chem 334:127428
83. Linares-Pasten JA, Aronsson A, Karlsson EN (2018) Structural considerations on the use of endo-xylanases for the production of prebiotic xylooligosaccharides from biomass. Curr Protein Pept Sci 19:48–67
84. Palaniappan A, Antony U, Emmambux MN (2021) Current status of xylooligosaccharides: production, characterization, health benefits and food application. Trends Food Sci Technol 111:506–519
85. Muralikrishna G, Subba Rao MVSST (2007) Cereal non-cellulosic polysaccharides: structure and function relationship-An overview. Crit Rev Food Sci Nutr 47:599–610
86. Scheller HV, Ulvskov P (2010) Hemicelluloses. Ann. Rev. Plant Biol 61:263–289

87. Palaniappan A, Yuvaraj SS, Sonaimuthu S, Antony U (2017a) Characterization of xylan from rice bran and finger millet seed coat for functional food applications. J Cereal Sci 75:296–305
88. Wen P, Zhang T, Wang J, Lian Z, Zhang J (2019) Production of xylooligosaccharides and monosaccharides from poplar by a two-step acetic acid and peroxide/acetic acid pretreatment. Biotechnol Biofuels 12:87
89. de Mattos NR, Colodette JL, de Oliveira CR (2019) Alkaline extraction and carboxymethylation of xylans from corn fiber. Cellulose 26:2177–2189
90. Khat-Udomkiri N, Sivamaruthi BS, Sirilun S, Lailerd N, Peerajan S, Chaiyasut C (2018) Optimization of alkaline pretreatment and enzymatic hydrolysis for the extraction of xylooligosaccharide from rice husk. AMB Express 8:115–124
91. de Carvalho DM, Martínez-Abad A, Evtuguin DV, Colodette JL, Lindstrom ME, Vilaplana F, Sevastyanova O (2017) Isolation and characterization of acetylated glucuronoarabinoxylan from sugarcane bagasse and straw. Carbohydr Polym 156:223–234
92. Fu G, Yue P, Hu Y, Li N, Shi Z, Peng F (2018) Fractionation of DMSO-extracted and NaOH-extracted hemicelluloses by gradient ethanol precipitation from neosinocalamus affinis. Int J Pol Sci 2018:1–8
93. Kilpeläinen P, Leppänen K, Spetz P, Kitunen V, Ilvesniemi H, Pranovich A, Willför S (2012) BIOREFINERY. Pressurised hot water extraction of acetylated xylan from birch sawdust. Nord Pulp Paper Res J 27:680–688
94. Abdella A, Ramadan S, Hamouda RA, Saddiq AA, Alhazmi NM, Al-Saman MA (2021) *Paecilomyces variotii* xylanase production, purification and characterization with antioxidant xylo-oligosaccharides production. Sci Rep 11:16468
95. Davani-Davari D, Negahdaripour M, Karimzadeh I, Seifan M, Mohkam M, Masoumi SJ, Berenjian A, Ghasemi Y (2019) Prebiotics: definition, types, sources, mechanisms, and clinical applications. Foods 8:92
96. Sheu WHH, Lee IT, Chen W, Chan YC (2008) Effects of xylooligosaccharides in type 2 diabetes mellitus. J Nutr Sci Vitaminol 54:396–401
97. Akhtar MS, Swamy MK (eds) (2018) Anticancer plants: natural products and biotechnological implements. Springer Nature, Singapore
98. Amir A, Arif M, Pande V (2013) Purification and characterization of xylanase from *Aspergillus fumigatus* isolated from soil. Afr J Biotechnol 12:3049–3057
99. Basotra N, Joshi S, Satyanarayana T, Pati PK, Tsang A, Chadha BS (2018) Expression of catalytically efficient xylanases from thermophilic fungus *Malbranchea cinnamomea* for synergistically enhancing hydrolysis of lignocellulosics. Int J Biol Macromol 108:185–192
100. Elgharbi F, Hmida-Sayari A, Zaafouri Y, Bejar S (2015) Expression of an *Aspergillus niger* xylanase in yeast: application in breadmaking and in vitro digestion. Int J Biol Macromol 79: 103–109
101. Knob A, Beitel SM, Fortkamp D, Terrasan CRF, De Almeida AF (2013) Production, purification, and characterization of a major *Penicillium glabrum* xylanase using brewer's spent grain as substrate. Biomed Res Int 2013:728735
102. Kumar V, Shukla P (2018) Extracellular xylanase production from *T. lanuginosus* VAPS24 at pilot scale and thermostability enhancement by immobilization. Process Biochem 71:53–60
103. Wang SY, Hu W, Lin XY, Wu ZH, Li YZ (2012) A novel cold-active xylanase from the cellulolytic myxobacterium *Sorangium cellulosum* So9733-1: gene cloning, expression, and enzymatic characterization. Appl Microbiol Biotechnol 93:1503–1512
104. Brenelli LB, Figueiredo FL, Damasio A, Franco TT, Rabelo SC (2020) An integrated approach to obtain xylo-oligosaccharides from sugarcane straw: from lab to pilot scale. Bioresour Technol 313:123637
105. Guido ES, Silveira JT, Kalil SJ (2019) Enzymatic production of xylooligosaccharides from beechwood xylan: effect of xylanase preparation on carbohydrate profile of the hydrolysates. Int Food Res J 26:713–721

106. Amorim C, Silverio SC, Silva SP, Coelho E, Coimbra MA, Prather KL, Rodrigues LR (2018) Single-step production of arabino-xylooligosaccharides by recombinant *Bacillus subtilis* 3610 cultivated in brewers' spent grain. Carbohydr Polym 199:546–554
107. Seesuriyachan P, Kawee-ai A, Chaiyaso T (2017) Green and chemical-free process of enzymatic xylooligosaccharide production from corncob: enhancement of the yields using a strategy of lignocellulosic destructuration by ultra-high pressure pretreatment. Bioresour Technol 241:537–544
108. Faryar R, Linares-Pasten JA, Immerzeel P, Mamo G, Andersson M, Stålbrand H, Mattiasson B, Karlsson EN (2015) Production of prebiotic xylooligosaccharides from alkaline extracted wheat straw using the K80R-variant of a thermostable alkali-tolerant xylanase. Food Bioprod Process 93:1–10
109. Jnawali P, Kumar V, Tanwar B, Hirdyani H, Gupta P (2018) Enzymatic production of xylooligosaccharides from brown coconut husk treated with sodium hydroxide. Waste Biomass Valorization 9:1757–1766
110. Palaniappan A, Balasubramaniam VG, Antony U (2017b) Prebiotic potential of xylooligosaccharides derived from finger millet seed coat. Food Biotechnol 31:264–280
111. Reddy SS, Krishnan C (2016) Production of high-pure xylooligosaccharides from sugarcane bagasse using crude β-xylosidase-free xylanase of *Bacillus subtilis* KCX006 and their bifidogenic function. LWT-Food Sci Technol 65:237–245
112. Khangwal I, Nath S, Kango N, Shukla P (2020) Endo-xylanase induced xylooligosaccharide production from corn cobs, its structural features, and concentration-dependent antioxidant activities. Biorefin, Biomass Convers. https://doi.org/10.1007/s13399-020-00997-3
113. Zhu F, Prosser C, Zhu Y, Otter D, Hemar Y (2018) Enzymatic formation of galactooligosaccharides in goat milk. Food Biosci 26:38–41
114. Sihui P, Ning D, Junyan R, Zhengbiao G, Caiming L, Yan H, Li C, Tod PH, Zhaofeng L (2017) Maltooligosaccharide-forming amylase: characteristics, preparation, and application. Biotechnol Adv 35:619–632
115. Kazim ARS, Jiang Y, Li S, He X (2021) *Aspergillus nidulans* AmyG functions as an intracellular a-amylase to promote a-glucan synthesis. Microbiol Spectr 9:e00644–e00621
116. Azcarate-Peril MA, Roach J, Marsh A, Chey WD, Sandborn WJ, Ritter AJ, Savaiano DA, Klaenhammer TR (2021) A doubleblind, 377-subject randomized study identifies *Ruminococcus*, *Coprococcus*, *Christensenella*, and *Collinsella* as long-term potential key players in the modulation of the gut microbiome of lactose intolerant individuals by galactooligosaccharides. Gut Microbes 13:1957536
117. Wu Y, Pan L, Shang QH, Ma XK, Long SF, Xu YT, Piao XS (2017) Effects of isomaltooligosaccharides as potential prebiotics on performance, immune function and gut microbiota in weaned pigs. Anim Feed Sci Technol 230:126–135
118. Gao J, Li X, Zhang G, Sadiq FA, Simal-Gandara J, Xiao J, Sang Y (2021) Probiotics in the dairy industry-advances and opportunities. Compr Rev Food Sci Food Saf 20:3937–3982
119. Wu H, Chiou J (2021) Potential benefits of probiotics and prebiotics for coronary heart disease and stroke. Nutrients 13:2878
120. Alvarez-Mon MA, Ortega MA, García-Montero C, Fraile-Martinez O, Monserrat J, Lahera G, Mora F, Rodriguez-Quiroga A, Fernandez-Rojo S, Quintero J, Alvarez-Mon M (2021) Exploring the role of nutraceuticals in major depressive disorder (MDD): rationale, state of the art and future prospects. Pharmaceuticals 14:821
121. Beam A, Clinger E, Hao L (2021) Effect of diet and dietary components on the composition of the gut microbiota. Nutrients 13:2795
122. Grela ER, Swiatkiewicz M, Florek M, Bakowski M, Skiba G (2021) Effect of inulin source and a probiotic supplement in pig diets on carcass traits, meat quality and fatty acid composition in finishing pigs. Animals 11:2438
123. Chlebicz-Wójcik A, Śliżewska K (2020) The effect of recently developed synbiotic preparations on dominant fecal microbiota and organic acids concentrations in feces of piglets from nursing to fattening. Animals 10:1999

124. Birmani MW, Nawab A, Ghani MW, Li G, Xiao M, An L (2019) A review: role of inulin in animal nutrition. J Food Technol Res 6:18–27
125. Jana UK, Suryawanshi RK, Prajapati BP, Soni H, Kango N (2018) Production optimization and characterization of mannooligosaccharide generating β-mannanase from *Aspergillus oryzae*. Bioresour Technol 268:308–314
126. Gainza O, Romero J (2020) Effect of mannan oligosaccharides on the microbiota and productivity parameters of *Litopenaeus vannamei* shrimp under intensive cultivation in Ecuador. Sci Rep 10:2719
127. Ben Ali M, Mhiri S, Mezghani M, Bejar S (2001) Purification and sequence analysis of the atypical maltohexaose-forming alpha-amylase of the *Bacillus stearothermophilus* US100. Enzym Microb Technol 28:537–542
128. Chen X, Wang J, Li Q (2015) Simultaneous determination of maltooligosaccharides in beer using HPLC-ELSD and their influence on beer foam stability. J Am Soc Brew Chem 73:78–83
129. Zheng CJ, Liu R, Xue B, Luo J, Gao L, Wang Y, Ou S, Li S, Peng X (2017) Impact and consequences of polyphenols and fructooligosaccharide interplay on gut microbiota in rats. Food Funct 8:1925–1932
130. Crittenden RG, Playne M (1996) Production, properties and applications of food-grade oligosaccharides. Trends Food Sci Technol 7:353–361

Applications of Fungi for Alternative Protein

13

Ryan Huling

Abstract (A Smarter Way to Make Protein)

Fungi are among the most versatile organisms on Earth, with innovative applications in all three pillars of the emergent alternative protein sector—plant-based, fermentation-enabled, and cultivated proteins. Just like the diversity of fungi, the variety of food uses continues to grow exponentially in all directions, now including fungi-based flavoring agents, yeast-based proteins, cultivated meat scaffolds, and even whole-cut alternative meats—thick, three-dimensional meat products that biomimic animal products like steaks and chicken breasts—created through solid-state fermentation. This versatility creates lucrative opportunities for innovative startups, scientists, and legacy food brands to develop products and technology platforms that capitalize on one of the fastest-growing food industry categories.

India's role as a world-class bio-manufacturing and technology hub makes it uniquely well-positioned to dominate these new fungi protein production categories if key stakeholders from academia, government, and the private sector work collaboratively to align their ambitions and resolve industry bottlenecks.

As the world grapples with the twin challenges of skyrocketing population growth and diminishing natural resources, harnessing fungi's potential to produce delicious and affordable alternative meat, dairy, and egg products could almost single-handedly resolve the global protein deficit without depleting the oceans or chopping down the rainforests in the process. It's simply a smarter way to make protein.

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Keywords

Fungi-based protein · Mycoprotein · Solid-state fermentation · GHG emissions · MycoTechnology · Quorn · Texturization

13.1 Introduction

In the 30-year span between 1989 and 2019, global meat production nearly doubled—from 174 to 337 million tonnes—driven in large part by emerging economies [1]. The most substantial growth in meat consumption per capita has occurred in Asia, particularly in China.

The forecast for the future is even more dire. As shown in the figure below, under a business-as-usual scenario, economic growth and rising incomes are expected to drive Asia's appetite for conventional meat and seafood to increase by 78% from 2017 to 2050 (Fig. 13.1) [2].

These dramatic increases have manifold negative impacts on our planet. Beef production is the top driver of deforestation globally, with cattle ranching directly associated with 80% of current Amazon deforestation. Animal-based agriculture is responsible for between 20 and 33% of all freshwater consumption in the world, surpassing the 19% used for industrial water withdrawal and the 11% for all municipal and household purposes. Global greenhouse gas (GHG) emissions by the livestock sector alone exceed emissions by all cars, trains, ships, and airplanes in the world combined and contribute more than the overall GHG emissions of the United States.

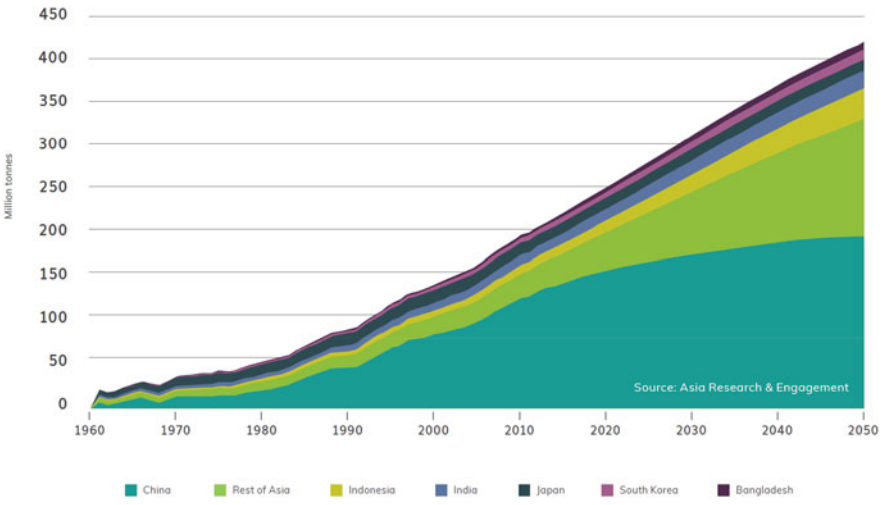

Fig. 13.1 Asia's projected meat and seafood consumption growth, 1961–2050

In a 2021 report titled *The Need for Change: Impact of Animal-based Diets on Natural Resources, Climate Change, and Human Health*, Dr. Albert T. Lieberg, a senior advisor to the United Nations, warned that "Our collective overreliance on resource-intensive foods—particularly those produced from animals—has strained the viability of vital ecosystems and the capacity of our limited natural resources in a potentially irreversible way, and contributed to a skyrocketing global obesity rate and serious medical concerns that jeopardize humans' ability to live healthy, productive lives. We cannot afford to continue externalizing the negative impacts of current food systems and related dietary patterns, or perpetuating practices that knowingly degrade the air, water, and land we all rely on. Rather, we must embrace the technological developments taking place within the food sector that will allow both our communities and industries to thrive for the long term." [1]

Fungi-based proteins play a central role in building a more robust global food system that is better equipped to handle the escalating pressures of skyrocketing protein demand, increased climate disruption, and threats of viral outbreaks.

Nutrient-dense ingredients like mycoprotein—produced through biomass fermentation—leverage the fast growth of microorganisms to efficiently produce large quantities of protein. The microbial biomass itself serves as an ingredient with the cells intact or minimally processed to a protein concentrate. Producing protein through this method carries many advantages over traditional crop agriculture. For example, while crop plants typically require growing seasons of weeks or months, the doubling time of most microorganisms is hours or even minutes. Fermentation capitalizes on the fundamental biological property of exponential growth, meaning that every growth cycle doubles the available biomass. When performed at the scale of hundreds of thousands of liters, these processes generate tens of tonnes of biomass every hour.

As population growth and urban sprawl continue to deplete available arable land, fungi offer an opportunity to produce sufficiently large volumes of protein to meet rising consumer demand while depleting a tiny fraction of water and land required for conventional animal protein. Indeed, many fungi that naturally lend themselves to replicating the taste and culinary experience of meat, like lion's mane mushroom, require no farmland at all, because they can be grown vertically in densely populated cities. Similarly, mycoprotein is grown in vats, thereby liberating its production from any traditional agricultural and geographical constraints [2].

Despite these promising attributes, market success for fungi-based proteins is by no means inevitable. Compared to investments in other ascendant sectors of the economy, like renewable energy and transportation, the food sector has received a comparatively tiny amount relative to its potential climate impact [3]. Substantial funding from both the public and private sectors—specifically geared toward open-access scientific R & D—is needed to unlock fungi's potential as an alternative protein source.

In this chapter, we will outline both the known food applications of fungi-based proteins and spotlight areas where further scientific research could break through industry bottlenecks and deliver the delicious and affordable proteins needed to reliably sustain our growing population.

13.2 Traditional Food Applications of Fungi

Fungi have long been consumed for both food and medicinal purposes, with records of various applications in ancient Egyptian murals and Greek writings and documentation in Chinese and Japanese traditional medicine. Fungi-based fermentation products like soy sauces, tempeh, sake, koji, oncom, angkak, miso, and hamanatto have been widely used throughout Asian cuisines. In western countries, preparation of mold-ripened cheeses like camembert, brie, roquefort, and blue stilton cheese, as well as fermentation of leavened bread using yeast, has been among the more common fungal applications. Instances of cereal fermentation using a mixed culture of *Lactobacillus* and yeast to produce beers and fermented porridges can be found in African traditional culture [4]. The production of edible mushrooms—including button mushroom, shiitake, Chinese or straw mushroom, winter mushroom, oyster mushroom, and truffles—is a common practice observed around the world.

Broadly speaking, fungal fermentation can be divided into three categories:

1. Natural fermentation: Uncooked ingredients undergo fermentation without the addition of any external fungal culture. Commonly known foods include idli and naan. The fermentation occurs due to the presence of microbes in the ingredients or the utensil surfaces.
2. Starter-mediated single-stage fermentation: In this case, the ingredient or substrate is cooked, and starter media in the form of spores or mycelium is added to it to initiate the fermentation process. The fermented products are cooked before consumption. Indonesian staples like tempeh and oncom are produced by this method.
3. Multi-stage fermentation: In this case, solid-state fermentation—where microbes are inoculated onto a moistened solid feedstock that may be enclosed or even grown in open air, leading to the production of digestive enzymes—is followed by liquid-state or solid-state fermentation involving the breakdown of carbohydrates and proteins. Typical products made from this type of fermentation include rice wine, soy sauce, and vinegar.

Building on the knowledge gained through traditional food applications, a new ecosystem of fungi-based alternative proteins has emerged with the potential to perform a similar dietary role to conventional animal proteins on consumers' plates.

Fermentation as a platform can be utilized in three ways for applications in the alternative protein space. The first route is through traditional fermentation in which fungi are used to ferment a target substrate to enhance its sensorial or textural properties for improved product properties. The second route is biomass fermentation to produce whole biomass like filamentous fungi and yeast for proteins, flavors, or textured alternative meats. The third route is precision fermentation, which generally uses recombinant DNA technology to exploit fungi as a platform for production of highly specific compounds. This chapter focuses on fungi-based alternative protein applications using the first two fermentation routes.

13.3 Applications of Fungi for Use in Alternative Proteins

Alternative proteins are designed to replace conventional meat, seafood, eggs, and dairy by competing on taste and price. To date, no alternative protein product both tastes the same as animal protein or better and costs the same or less. Yet the pace of food technology innovation has been impressive, scaling up along a similar trajectory to electric cars or renewable energy and consistently improving on overall taste quality. As a result, alternative proteins are increasingly attractive to consumers, and fungi are poised to play a critical role in that shift.

13.3.1 Traditional and Solid-State Fermentation Using Mushroom Mycelium

One of the most promising applications of solid-state fermentation for fungi-based alternative meat products is the creation of whole-cut meats from mushrooms.

Given the range of mushroom species available, food producers can be highly selective in picking ingredients that naturally lend themselves to replicating the flavor of conventional animal proteins. *Laetiporus sulphureus*, for example, which has been nicknamed the "chicken of the woods" is popular for its chicken-like taste when cooked. *Hericium erinaceus*, also known as lion's mane mushroom, is known for its close resemblance to crab and other seafoods. *Hypomyces lactifluorum*, also known as lobster mushroom, tastes similar to its namesake crustacean. [Lobster mushroom is, in fact, not a mushroom, but a parasitic ascomycete fungus that feeds on other mushroom species and distorts their shape and color.] *Pleurotus ostreatus*, commonly known as oyster mushroom, is known for tasting like scallops and bacon upon cooking. *Russula xerampelina*, also known as the crab brittlegill or shrimp mushroom, and *Entoloma abortivum*, nicknamed the "shrimp of the woods" have a taste profile similar to shrimp.

Given their taste and textural similarities, it's possible to scientifically map the various edible mushroom species that mimic meat and seafood and evaluate the feasibility of using both traditional mushroom fruiting body cultivation techniques and modified solid-state fermentation to grow mushroom mycelium for whole-cut alternative meat and seafood products. Leveraging culinary expertise from chefs would also aid species selection and development of post-processing techniques to create mushroom-based products.

As an example, Atlast Food Co., a spinout from materials company Ecovative, is utilizing solid-state fermentation to produce whole-cut meats from mushroom mycelium like those shown below. Other companies like Savory Wild, Pan's Mushroom Jerky, and Jewels of the Forest are selling mushroom jerky as substitutes for conventional meat. While the market for mushroom jerky products is relatively small compared to whole-cut meats, these products can help build consumer awareness about the versatile applications of mushrooms in the alternative protein sector (Fig. 13.2).

Fig. 13.2 Mycelium bacon, produced by Atlast Food Co

Other startups developing whole-cut meats using fungi include Meati (steak and cubed chicken breast) and Aqua Cultured Foods (whole-cut fish). Both are currently focused on building up their production capacity and do not yet have products widely available in the market.

In another instance, Fotortec, a Chile-based startup, has created several ingredients from oyster mushrooms with different applications in the alternative protein sector. F1 flavor enhancer is one such ingredient made out of dehydrated edible mushrooms which amplifies the umami flavor without drastically impacting the sodium content of a food product. Other ingredients that Fotortec is exploring include a texture enhancer and mushroom-derived protein isolate, which can be used for creating desired alternative protein products.

Fungi can also play a central role in mitigating the existing constraints of common alternative meat ingredients. Plant-based protein sources like soy and pea protein suffer from the disadvantage of retaining off-flavors, which connote beany and green tastes due to the presence of aldehydes, ketones, furans, and alcohols. Fermentation is one approach to reduce these off-flavors. Drs. Cynthia El Youssef and Pascal Bonnarme have demonstrated that when used to ferment pea proteins, a combination of lactic acid bacteria with various yeast species including *Kluyveromyces lactis*, *Kluyveromyces marxianus*, and *Torulaspora delbrueckii* led to a significant reduction in green off-flavor notes as indicated by a sensory evaluation [5]. American startup MycoTechnology has also used shiitake mycelium to ferment a blend of pea and rice protein to improve the flavor, functionality, bioavailability, and nutrition of the plant-based protein.

13.3.2 Submerged Fermentation for Filamentous Fungi Biomass: The Quorn Story

While filamentous fungi are known to produce a wide range of products like antibiotics, organic acids, enzymes, and heterologous proteins, the most commonly known food application is the protein-rich product Quorn, marketed by Marlow Foods. The key advantage of using filamentous fungi is the ability to utilize the whole biomass for creating an alternative meat product using simple processing techniques that don't rely on traditional texturization technology like extruders.

Case Study: Production of Filamentous Fungi Under the Quorn Brand

In the late 1950s, there was growing interest in single-cell protein sources due to the anticipated shortage of protein-rich foods by the 1980s. The lower cost associated with manufacturing, ease of harvesting and scale-up, and desirable essential amino acid profile made filamentous fungi an attractive option for exploration. Compared to other microorganisms, filamentous fungi were found suitable for further research because of the demonstrated use of *Rhizopus oligosporus* species in foods such as tempeh and the desirable organoleptic properties making it appropriate for food applications. *Fusarium venenatum* A3/5 was the first filamentous fungus used to create alternative meats. The development of filamentous fungi as a source of protein

by the British company Rank Hovis McDougall (RHM) began in the 1960s. The strain was screened from a pool of 3000 different fungi over 3 years. It took 12 years of intensive research and testing to establish that the strain was safe for human consumption and to get approval from the Ministry of Agriculture for utilization of *Fusarium venenatum A3/5* as food in 1984. Various tests carried out to secure approval included toxicology testing, animal feeding trials, human trials with 2500 volunteers, and storage stability. No adverse effects on animals or immunological responses among humans were found. The UK Food Standards Committee coined the term "mycoprotein" as a generic name for food products derived from filamentous fungi. The filamentous fungi-based products from Marlow Foods are labeled under the brand name Quorn. Additional toxicological testing of Quorn products by AstraZeneca was carried out in 1996 to secure approval from the US Food and Drug Administration for the sale of Quorn products in the United States [6]..

The manufacturing process of Quorn products involves several steps that in combination help align and bind the fibers of mycoprotein together. To develop the characteristic texture of Quorn products, a series of steaming, chilling, and freezing processes are employed. Mycoprotein is made in fermenters more than 40 m high, which run continuously for at least 5 weeks at a time. For the production of filamentous fungi on a large scale, a joint venture between RHM and Imperial Chemical Industries (ICI) was established under the name Marlow Foods Limited. While RHM had worked on developing the product, ICI provided the manufacturing facility including the fermenters.

Manufacturing Process

From a manufacturing standpoint, several configurations of liquid-state fermentation setups were evaluated for *Fusarium venenatum*. Batch and fed-batch processes have already been used at an industrial scale for the production of various high-value products such as enzymes, antibiotics, and other pharmaceutical compounds. However, a continuous flow process was preferred for the production of *Fusarium venenatum* biomass due to the higher productivity and convenience of running the fermentation process up to 6 weeks.

Up until 1994, a $40m^3$ airlift fermentor designed by ICI for growing bacteria was utilized for the production of *Fusarium venenatum* biomass with a capacity of 1000 tonnes of annual production of Quorn mycoprotein. Subsequently, two $155m^3$ airlift fermenters were built, increasing the production capacity to 10,000–14,000 tonnes per annum [7].

To start the process, the fermenter is inoculated with 50 g of biomass in 5 L of batch culture. The continuous production starts 4 days after inoculation. The airlift fermenter is designed in such a way that the gaseous phase, including ammonia and sterilized air, is input through a riser at the bottom of the fermentor. The gaseous phase is dispersed in the culture in the form of bubbles, which allow for efficient mass transfer. As the gaseous phase rises with the culture, the oxygen concentration reduces, and CO_2 concentration increases. At the top of the fermenter, CO_2 is released due to low pressure, and culture enters the downcomer where it is redirected to the riser and the circulation of hyphal filaments continues due to the relative

difference in densities between the culture in the downcomer and the aerated culture in the rising section. Since this is a continuous process, the biomass is removed after every 5–6 h.

Process Conditions and Nutrient Media

The nutrient media required for the growth of filamentous fungi includes nitrogen and oxygen from sterilized compressed air, ammonia, glucose, biotin, minerals, and other trace elements. All nutrients need to be sterilized to prevent contamination. The pH is maintained at 6.0 and ammonia flow is controlled to maintain this pH. The temperature range of 28–30 degrees Celsius is also maintained through a heat exchanger with external cooling coils to remove heat generated during biomass production. The CO_2 evolution rate indicates the biomass concentration and, thus, can be used to control the nutrient flow rate. The nutrient solution or the liquid phase is fed at a dilution rate in the range of 0.17–0.2 $h.^{-1}$ to achieve the target maximum growth rate of 10–15 g/l. [7] Here, glucose is maintained in excess. The growth rate is maintained in this range to prevent the production of toxic secondary metabolites. Since this is a continuous fermentation process, the product is harvested continuously.

Post-processing and Harvesting

Before harvesting the biomass, it is necessary to bring down the nucleic acid content. As per WHO recommendation, human ingestion of RNA from single-cell protein sources should be less than 2 g RNA per day and less than 4 g total nucleic acid per day. The RNA content in filamentous fungi is in the range of 8–9%, which limits the daily consumption of biomass to 20 g. To overcome this limitation, the biomass is subjected to thermal shock by raising the temperature to 68 degrees Celsius for 20–30 mins, leading to disruption of ribosomes, activation of endogenous ribonuclease, and subsequent breaking down of RNA to nucleotides, which get transferred to the culture broth through diffusion. The RNA reduction process leads to 1% (w/w) concentration of RNA and also leads to loss of protein.

Due to the filamentous nature of *Fusarium venenatum*, it is much easier to harvest compared to other single-cell protein sources like bacteria and yeast. After the thermal shock and diffusion of RNA into culture broth, mycelium suspension with an initial concentration of 1.5% (w/w) solids is heated to 90 degrees Celsius and centrifuged to get to a concentration of 20–30 percent (w/w) solids. The high-temperature extraction does not impact the fibrous texture or organoleptic properties of the products [8]. The pasty material obtained after harvesting is chilled to 4 degrees Celsius and undergoes a mechanical process leading to the alignment of filaments of fungi to form a fibrous structure. Other ingredients for color, flavor, and binding to stabilize the aligned fibers are added, and the resulting material is steam-cooked for 30 mins, then chilled. Depending on the final product, the material can be shaped using traditional food processing equipment and frozen for storage. Freezing plays an important role as the ice crystals bring the fibers closer to create bundles giving the product a meat-like texture.

Initially, Quorn products were simply marketed as a healthy food with low fat, no cholesterol, and high dietary fiber. However, owing to the texture of Quorn products and early evidence from consumer surveys in the 1990s indicating that the UK population was moving toward lower red meat consumption, Marlow Foods started positioning Quorn as a meat replacer with a product range that includes mince, chicken-style pieces, sausages, burgers, and ready-made meals.

13.3.3 Application of Fungal Biomass for Cultivated Meat

Cultivated meat is genuine animal meat (including seafood) that is produced by cultivating animal cells directly. This production method eliminates the need to raise and farm animals for food.

While filamentous fungi have obvious applications in the alternative protein sector, there are also emerging opportunities for mycelium-based scaffolding materials and production of flavoring and stabilization agents.

Scaffolding provides structural support for cells to adhere, differentiate, and mature, making it crucial for the creation of structured meat products like steak. Approaches for utilizing fungi for the development of scaffolding materials include the use of chitin/chitosan derived from the fungal cell wall. The use of chitosan-based scaffolds has been demonstrated to support myoblast adherence and differentiation; it can form a scaffold with mechanical properties compatible with meat-relevant cells and is already used in edible coatings in the food industry [9].

Chitosan is formed by the alkaline deacetylation of chitin which impacts the physical, chemical, and biological properties of scaffolds [10]. The degradation of chitosan depends on the degree of deacetylation. This property can be used to customize scaffolding material used as per desired application. Biodegradable chitosan with a controlled degradation rate can act as a temporary matrix for cell adhesion [11–13]. Thus, chitin or chitosan-based scaffolds can be a potential substrate for cultivated meat products. Dr. Natalie Rubio has described chitosan as a promising scaffolding material for cultivated seafood production in particular, as it is edible, is well-referenced in tissue engineering research, is an accessible biopolymer, and can be cast as membrane, hydrogels, and sponges [14, 15]. Also, fungal chitosan is non-allergenic and approved for human consumption as a food additive or nutrition supplement [16–18].

While growing cells on chitosan is possible, the lack of recognition motifs that signal cell adhesion and migration limits their functionality. However, this limitation can be overcome by modifying the properties of chitosan by blending it with other polymers to resemble native tissue [19].

The majority of chitin production relies on by-products from the shellfish industry. Thus, there is an opportunity to leverage fermentation platforms to obtain scalable production of non-animal fungi-based chitin, which can then be utilized for cultivated meat applications. Identifying species and strains that can produce high quantities of chitin and developing appropriate downstream processing steps to

valorize different fungal components including chitin will play a key role in bringing down the cost of fungi-derived chitin and demonstrating the viability of animal-free chitin production systems for cultivated meat applications. Chitin derived from *Aspergillus niger* and *Pleurotus ostreatus* is already sold in the market. Fungi-derived chitin also has a key advantage of being allergen-free compared to crustacean-derived chitin.

Fungal mycelium can also be utilized for building the scaffolding matrix, because its textural profile can provide a desired mouthfeel and umami flavor associated with meat. Excel™ Scaffolding, created by Ecovative's Mycelium Foundry One, is an example of an edible cell scaffold that can be used for the development of whole-cut, cultivated meat products. Mycelium is used to grow an edible 3D matrix of micro-structure matrices to support the adhesion of cells and the growth of cells into differentiated tissues. Similarly, India-based startup Myoworks is developing an edible scaffolding technology from mycelium for cell adhesion, growth, and proliferation for cultivated meat applications. More open-access research focused on exploring various species and strains optimized for creating edible scaffolding materials from fungal mycelium would help accelerate the development of cultivated meat and seafood products.

13.3.4 Yeast as a Source of Single-Cell Protein (SCP)

There are very few companies focused on using single-cell proteins from yeast for alternative protein applications. However, due to the prevalent use of yeast in the alcoholic beverage and baking industries, yeast as a microorganism is not only a source of protein but also comes with years of established knowledge on production through fermentation with existing infrastructure. For alternative protein applications, yeast biomass or protein isolates with a neutral taste, color, and odor obtained by choosing the right strains from existing as well as novel strains created through breeding techniques will be a critical step for the development of the fungi value chain. With fairly well-established production systems and downstream operations, the focus on strain selection and process optimization for strains of interest will enable the utilization of yeast-based ingredients for the alternative protein sector.

There are several advantages of using single-cell biomass like yeast as a source of protein compared to traditional sources of food, especially animal-based sources [20], including:

1. Ability to manipulate microbial quantity as well as a composition by using different strains, species, nutrient media, and processing parameters
2. High efficiency of substrate conversion
3. High productivity or growth rate of biomass
4. No dependence on seasons and variation in atmospheric conditions

Low cell wall digestibility and high content of nucleic acid are the key areas that need to be addressed for making yeast-based proteins suitable for human

consumption. Compared to bacteria, yeast is easier to harvest due to its larger size and has lower nucleic acid content, higher lysine content (an essential dietary amino acid), and the ability to grow in acidic pH. Yeast also has wider acceptance in food applications for human consumption due to traditional usage in the baking industry.

13.4 Overview of the Fungal Value Chain for Alternative Protein

Depending on the application area, the value chain for utilization of fungi-derived ingredients will differ. However, there are three key steps involved in the production of fungi and subsequent utilization of fungi-based ingredients irrespective of end application. The process can be broken down into three broad categories: strain selection involving screening of suitable strains, production involving biomass production in manufacturing scale while maintaining sterile conditions, and downstream processing involving destruction of RNA or other toxic compounds, harvesting, and concentration of biomass followed by extraction of target compounds. These have been described in the chart below (Fig. 13.3).

The specific steps involved in the production of yeast, filamentous fungi, and mycelium as sources of protein are similar in nature. Other ingredients like enzymes and chitin can be extracted downstream, while the upstream production steps remain the same. The choice of technology at each stage of the value chain will differ due to

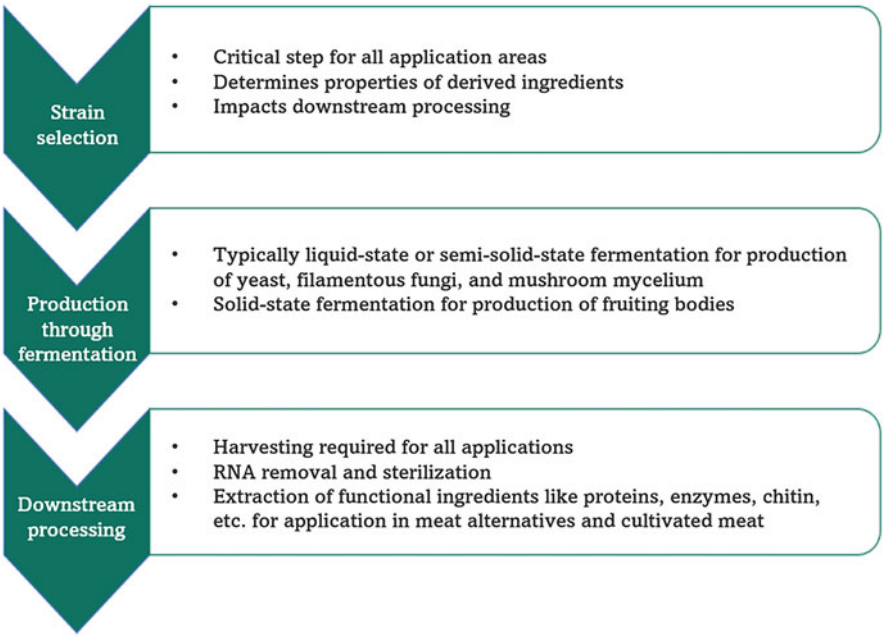

Fig. 13.3 Typical steps involved in the production of fungi-based ingredients

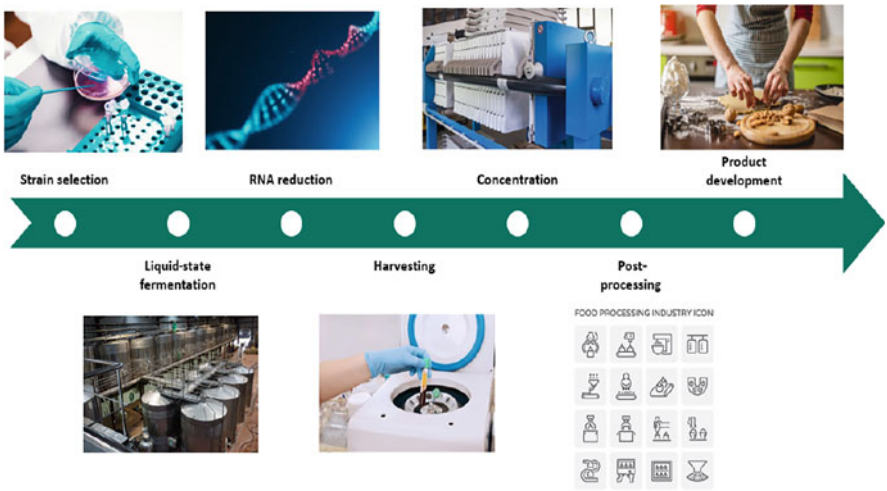

Fig.13.4 The value chain for fungal production using liquid-state or semi-solid-state fermentation

the differences in the morphological and metabolic properties of the yeast, filamentous fungi, and mycelium. For example, baker's yeast is typically produced in a fed-batch fermentation system running up to 20 h, while filamentous fungi like *Fusarium venenatum* are produced in a continuous fermentation system running for a period of 6 weeks (Fig. 13.4). Even though both species of fungi can be produced in a liquid-state fermentation, the configuration and type of bioreactors used may differ.

Comparatively, the rules of scaling up or scaling out production systems for solid-state fermentation are not as well-established as in the case of liquid-state fermentation. The value chain for solid-state fermentation has been depicted below (Fig. 13.5):

Fig. 13.5 The value chain for fungi production using solid-state fermentation

13.5 Nutritional Benefits of Fungi-Based Proteins

For fungi-derived proteins to compete with existing protein sources, the nutritional profile, digestibility, and functionality of these proteins need to be either the same or better than animal- or plant-based proteins. A key indicator of protein quality is its amino acid profile. Since not all amino acids are produced in the human body, the essential amino acids (EAA) need to come from external protein sources like food. The protein quality from fungi depends on various intrinsic and extrinsic factors such as choice of fungal strain, production system, growth media and nutrients supplied, and downstream processing.

Depending on the species used, fungi contain a protein content ranging from 15 to 50% on a dry weight basis, all essential amino acids, high dietary fiber coming from the chitinous cell wall, high vitamin B content, low fat, and no cholesterol. The crude protein content in edible mushrooms also varies depending on their stage of development [21].

Proteins in edible mushrooms are rich in glutamic acid, aspartic acid, and arginine. The reported essential amino acid profiles of edible mushrooms also show that mushrooms have a relatively lower content of sulfur-containing amino acids like methionine and cysteine but are rich in threonine and valine [22]. Though the free amino acid content in dried edible mushrooms is low, the presence of aspartic acid and glutamic acids is responsible for their umami flavor [23]. Additionally, mushrooms are a good source of vitamins such as riboflavin, niacin, and folates. The vitamin B2 content in mushrooms is higher compared to vegetables, and some varieties of *Agaricus bisporus* have content as high as conventional eggs and cheese [24].

When screening strains and optimizing production systems for fungi, the amino acid profile should be targeted such that it meets the FAO reference standards on the recommendation of essential amino acid content in a protein mix. It would be advantageous to have amino acid profiles superior to proteins derived from eggs and soy. This can be evaluated using the essential amino acid index (EAAI), which relates the content of each essential amino acid in a protein to the amino acid content in egg protein.

Apart from the amino acid profile, protein bioavailability and digestibility are crucial parameters that indicate the absorption of nutrients in the human body. The protein digestibility-corrected amino acid score (PDCAAS) is an index recommended by FAO in 1989 (Table 13.1). The nutritional profiles of edible filamentous fungi-based products like Quorn and Promyc (a fungi-based and fermented ingredient produced by Mycorena) are available in the public domain (Table 13.2).

The PDCAAS for mycoprotein as reported in scientific literature is 0.996, which is close to that of chicken and milk. Mycoprotein also contains all essential amino acids, making it a complete protein source [25]. The bioavailability of mycoprotein has been demonstrated to be similar to that of milk protein and significantly better than wheat-based and soy-based protein. This is a compelling contrast to draw given

Table 13.1 Comparison of bioavailability and essential amino acid profiles of various protein sources

Protein source	PDCAAS	Complete EAA
Mycoprotein	0.99	Yes
Chicken	1.00	Yes
Eggs	1.00	Yes
Milk	1.00	Yes
Beef	0.92	Yes
Soy	0.91	Yes
Pea	0.89	Yes
Fish	1.00	Yes
Hemp	0.61	Yes
Beans	0.68	No
Chickpeas	0.78	No
Cashew nuts	0.90	No
Wheat	0.42	No
Rice	0.44	No

Table 13.2 Nutritional composition of mycoprotein per 100 g (wet weight)

	Promyc	Quorn	
Energy	350	360	kJ
Protein	15.07	11.50	g
Energy	85	86	kcal
Fiber	3.08	6	g
Fat	1.49	2.90	g
Saturated fat	0.34	0.60	g
Monounsaturated fat	0.40	0.50	g
Polyunsaturated fat	0.68	1.80	g
Carbohydrates	0.94	1.70	g
Sugar	<0.30	0.80	g

that wheat-based and soy-based proteins are the baseline standard among plant-based meats and mycoprotein carries distinct advantages over them.

According to a recent study conducted on a group of healthy young men, the ingestion of 60 g of mycoprotein created an optimal blood amino acid response for muscle protein synthesis [26].

Mycoprotein also contains high dietary fiber composed of polymeric n-acetyl glucosamine (chitin) and beta 1–3 and 1–6 glucans, in the range of 12–20% by dry weight. Studies have demonstrated that consumption of this type of fiber can provide relief from joint pain in osteoarthritis, stimulate beneficial bacteria in the colon, and improve glycemic profile [27–29]. In addition to this, mycoprotein fiber can impact satiety and lower cholesterol. According to a study with a test group, individuals who consumed mycoprotein for lunch ingested up to 24% fewer calories in dinner the same day and the following day. This feeling of fullness after a mycoprotein consumption is attributed to the protein and fiber content in mycoprotein. Hence, a mycoprotein diet can impact hunger and total calorie intake making weight loss easier [27, 30–32].

13.6 Marketing the Nutritional Benefits of Fungi-Based Protein

The vast majority of consumers who purchase alternative proteins—up to 98% in markets like the United States—also purchase conventional meat products [33]. Consumer studies by Mintel and other research firms show that one of the primary drivers of such "flexitarian" purchases is the desire "to be healthier." [34]

This motivation to consume healthier foods creates favorable conditions for innovative fungi-based proteins to thrive, but only if they're advertised in a strategic way. While consumers do want to make healthy choices, they don't want to feel like they will be sacrificing on flavor to do so. Walking the line between emphasizing the positive attributes of fungi-based proteins while also avoiding language that could be interpreted as restrictive (e.g., framing alternative proteins as "light and low carb") is critical to achieving market success.

Fortunately for fungi-based proteins, there are many positive attributes that can be emphasized to make this case:

- Taste is the attribute most likely to drive purchase intent for all age groups and diet types. Using positive, indulgent language that emphasizes taste and flavor is much more effective than positioning dishes as the "better" choices [35]. Positive taste connotations can be generated by a number of factors, including vivid, tempting imagery of the product on packaging; bright, saturated colors on a dark or light background; and visibility of the product through the packaging when it looks similar to its conventional meat counterpart, as many fungi-based proteins do [36].

- Familiarity and tradition are also strong positive drivers of purchase intent. Consumers are more likely to purchase products that appear familiar to them than those that appear novel. Omnivore consumers like products that look comparable to their conventional meat counterparts and language that isn't unusual or incongruous [36].
- Consumers say that protein is the most popular attribute in alternative meats, so spotlighting the fact that mycoprotein actually has a higher protein content percentage than many conventional meats like pork can demonstrate superiority over animal-based food products [2].
- As mentioned above, mycoprotein also contains essential amino acids and fiber, but is low in fat, making it a desirable food source for consumers trying to limit their fat intake while maintaining a high-protein diet [37].

It is recommended to sell fungi-based proteins in the same retail locations where conventional meat, dairy, and eggs are found. Studies have shown that 76% of consumers want to find alternative proteins in the conventional meat aisle and frozen area where they already shop [38]. This makes intuitive sense because the vast majority of alternative protein purchases are made by omnivorous consumers, so relegating products to a niche section aimed at health-conscious or vegetarian consumers will render them largely invisible to a receptive audience.

Similarly, altruistic benefits, such as improved animal welfare and lighter environmental impact of the products, are low in the importance hierarchy for omnivore consumers and unlikely to drive purchase intent. These considerations are more likely to appeal to millennials than to any other demographic group, but they are still less important than taste, familiarity, and health [38].

13.7 How the Alternative Protein Sector Could Boost Food Production in India and Deliver High-Quality Livelihoods for Local Producers

The traditional applications of fermentation at a household level discussed in earlier sections demonstrate that it is possible to create fungi-derived products at a small scale. Even today, cheese-making and wine-making operate at various scales, and small-scale local production is common for these processes. Kombucha preparation using a symbiotic culture of bacteria and yeast (SCOBY) at a household scale is a known practice. While liquid-state fermentation may require some degree of process control, solid-state fermentation as in the case of mushroom cultivation can be easily scaled out and facilitate income generation for small-scale producers.

Scaling down and decentralization of fermentation can be leveraged to not only cater to various application areas in the alternative protein sector but also meet nutritional needs in low-income, resource-scarce, and isolated regions where protein deficiency is a key concern due to limited access to high-quality affordable protein

sources. Fungi are an excellent choice for the production of proteins due to their ability to transform readily available carbohydrates (including simple sugar, of which India produces a glut) into proteins and provide biomass with all essential amino acids, high dietary fiber, high vitamin content, low fat, and no cholesterol.

Local production of fungi through small-scale decentralized units offers the flexibility to utilize a wide variety of feedstocks, including those coming from side streams of agriculture and food processing, as well as excess unsold produce. As a spillover effect, this will facilitate income generation for farmers and small-scale producers while also helping to meet the needs of the local community. For such a model to be successful, an upstream strategy needs to be developed to ease the process of technology adoption and knowledge transfer to local producers. Strains need to be optimized for local environmental conditions and available feedstock to obtain maximum productivity. Strains can also be optimized to meet the specific nutritional requirements of the community.

To ensure safe operation for a liquid-state fermentation unit, training for operating, maintaining, and cleaning small-scale bioreactors needs to be provided. The design of the setup should be such that it leads to no contamination and minimal human touchpoints for handling the biomass. Operating protocols need to adhere to good manufacturing practices. To ensure food safety and quality, training personnel for microbiological and chemical assessments is also critical. Recipes relevant to the community's culinary preferences should be co-developed to ensure the adoption of the food product by local consumers.

Mushroom cultivation is a technologically mature practice with policy support from the Indian government to not only train farmers but also provide subsidies. While scaling out protein production through mushroom cultivation is viable, such examples for liquid-state fermentation don't exist. Hence, the commercial viability and business model for scaling down liquid-state fermentation need to be explored further. However, given the immense potential of fermentation as a platform to meet nutritional needs in remote areas and protein-deficit communities, decentralization should be investigated as a preliminary step to make alternative proteins accessible and affordable to the bottom of the pyramid.

13.8 Snapshot of the Commercial Landscape for Fungi-Based Protein Cultivation and Production, Including B2B Producers of Protein Isolates

The diagram below shows some of the existing innovators utilizing fungi in the alternative protein sector for either business-to-consumer (B2C) or business-to-business (B2B) purposes (Fig. 13.6).

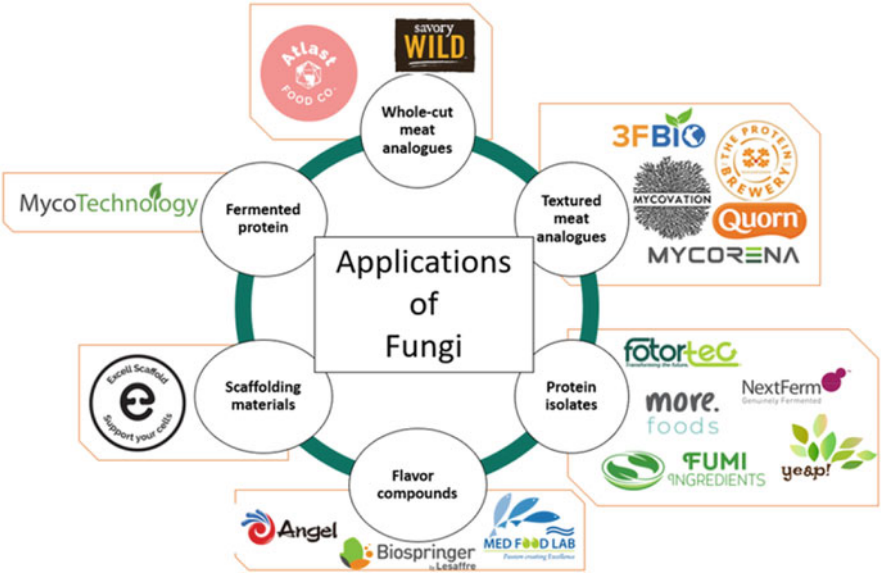

Fig. 13.6 Alternative protein applications of fungi

13.9 Notable Market Developments and Investments by Global Food Industry Leaders into Accelerating Fungi-Based Protein Production

Though Marlow Foods has historically dominated the utilization of filamentous fungi as an alternative meat technology, several other companies in this space have emerged in the last decade. One such company is ENOUGH (formerly known as 3F Bio) which is trying to address production costs to bring down the final product prices. In May 2021, Unilever, one of the world's largest food companies, forged a partnership with ENOUGH, declaring that "it's still early days but we see huge potential to apply [mycoprotein] technology to our portfolio across various ranges." [39]

To date, ENOUGH has developed a proprietary patented technology for the production of mycoprotein which brings down the production cost by half using an integrated, zero-waste fermentation process.

The integrated technology combines a first-generation biorefinery with traditional aerobic fermentation processes. The nutrient-rich by-product, distiller's dried grains with solubles (DDGS), is produced in the first-generation biorefinery where cereal-based feedstocks are converted to bioethanol (Fig. 13.7).

Currently, DDGS is sold as animal feed. ENOUGH has demonstrated mycoprotein production using grain starch hydrolysate, derived from cereals, which is used as feedstock for ethanol production. In the integrated process, about

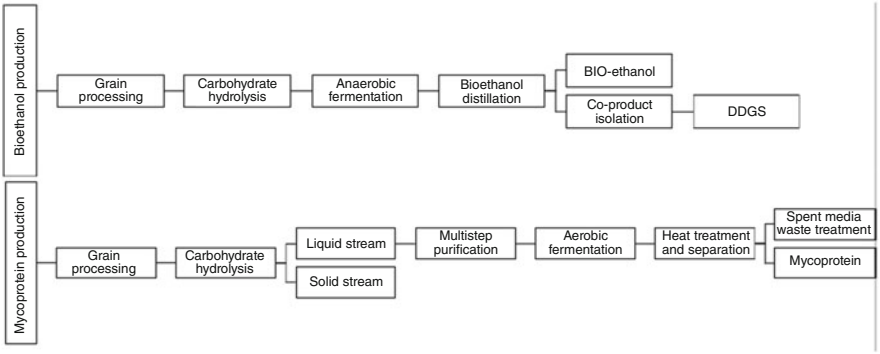

Fig. 13.7 Typical process for bioethanol and mycoprotein production

5–10% of the feedstock is segregated from biorefinery feed for use in the production of mycoprotein. Typically the carbohydrate-rich waste stream is discarded after aerobic fermentation. However, in the integrated process, the output stream with glucose and nutrients is recycled back to the feedstock stream creating a zero-waste technology (Fig. 13.8) [40].

This integrated process lowers the production cost by utilizing a low-cost feedstock and recycled waste streams, thus saving on effluent treatment costs and allowing proximity to existing biorefinery facilities [40]. It is known that greenhouse gas emissions associated with existing mycoprotein products are comparable to chicken, but this innovative integration process lowers that figure further by one third while also lowering the water and land footprint owing to high feed conversion efficiency and zero-waste process design. A key barrier to the expansion of mycoprotein products has been the limited expansion of production capacity across geographies. The integration of the mycoprotein production process with existing biorefineries reduces capital costs associated with expansion and diversification.

Two other companies utilizing fungi for alternative proteins include Mycorena and The Protein Brewery. As referenced earlier, Mycorena has created a neutral-tasting ingredient with a meat-like texture under the brand name Promyc. Promyc's technical datasheet shows that the ingredient has a high water holding capacity, comparable to pea and soy protein, and foaming and emulsification capacity. Thus, Promyc can have potential applications in smoothies, cream, mayonnaise, alternative meats and cheeses, and cakes. According to publicly available information, Promyc's production process uses starch-like carbon sources and has the flexibility to use side streams as feedstocks to enable a sustainable and cost-effective production system.

Mycorena also announced in November 2021 that they are launching a fungi-based fat ingredient designed to enhance plant-based foods [41]. The startup says that this new ingredient creates a profile of sensations similar to what consumers would expect from "a juicy steak," thus helping to take plant-based meats "to the next level."

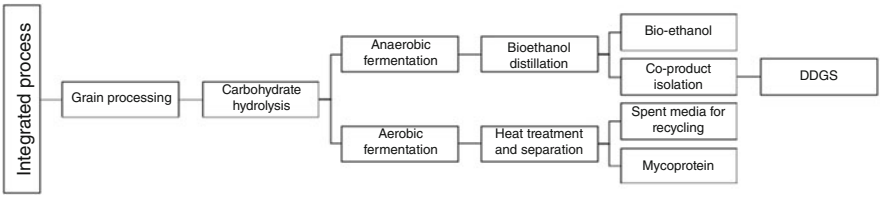

Fig. 13.8 Integrated process for co-manufacturing of bioethanol and mycoprotein, adapted from Ritchie et al. [40]

The Protein Brewery utilizes filamentous fungi for creating proteins and is focused on bringing down costs through feedstock optimization. The company has created a product named Fermotein, which has 47% protein content on a dry basis. The key differentiating factors for Fermotein include pleasant and less dry mouth-feel, which can be attributed to water-binding properties and unsaturated fat, in-built meat-like flavor due to presence of 10% fat, no off-taste, no chemical treatment or processing, and high nutritive value due to an amino acid profile close to meat and high fiber content.

Finally, in October 2021, global meat producer Hormel Foods (producer of the iconic canned meat Spam), entered into an exclusive partnership with fermented alternative protein startup The Better Meat Co. [42] Together, they are working to create new products using The Better Meat Co.'s signature mycoprotein ingredient, named Rhiza. The Better Meat Co.'s fermented alternative meats are not yet on the market, but as a proof of concept, the company announced a short test run at California steakhouse Bennett's American Cooking, which offered steaks made with Rhiza to its customers.

13.10 Market Developments and Investments in Yeast-Based Proteins

Biospringer Lesaffre, a global supplier of natural yeast extract and yeast-derived ingredients, has launched Springer Proteissimo 101, a yeast-derived protein with applications in alternative meat and cheese products. This product was launched in October 2020 in Europe, the Middle East, and Africa. Springer Proteissimo 101 has 75% protein content with a protein digestibility-corrected amino acid score (PDCAAS) of 1 and contains all essential amino acids. The key advantages of this product include its clean flavor without any off-notes, favorable melt behavior for creating cheese analogues, and its ability to improve chewability for creating alternative meats. Biospringer also offers yeast-based products that can impart chicken, beef, and cheese flavors in plant-based alternative meats and cheeses. The technical datasheet comparing the performance of yeast-derived proteins with pea protein and animal protein demonstrates the functional advantages of the yeast as a source of protein for alternative protein applications.

Similarly, Angel Yeast, a China-based manufacturer known for its yeast and yeast extract products, is exploring new yeast strains to target the emerging alternative protein market. One direct application of yeast extract in the alternative protein sector is that it can regulate the beany flavor and other off-notes of plant-based meat products while intensifying the meaty notes. The presence of nucleotides, amino acids, flavor peptides, and small protein molecules that undergo the Maillard reaction to produce nitrogen-rich and sulfur-rich compounds helps intensify the meaty flavor. While Angel Yeast is also looking into applications of yeast as a protein source for sports nutrition, there is scope to utilize their expertise in strain selection, yeast production, and flavor development for other applications in the alternative protein sector.

13.11 Snapshot of Capital Investment into Fungi-Based Protein Production to Date

Below is a summary of alternative protein investments focused on traditional or biomass fermentation, based on analysis of PitchBook Data by The Good Food Institute.

Investments comprise accelerator and incubator funding, angel funding, seed funding, equity and product crowdfunding, early-stage venture capital, late-stage venture capital, private equity growth/expansion, capitalization, corporate venture, joint venture, convertible debt, and general debt completed deals (Table 13.3).

13.12 Anticipated Challenges in Using Fungi-Based Proteins for Alternative Meats

The utilization of fungi for alternative protein requires advancements across the value chain from strain selection to end product development. Irrespective of application areas, the ecosystem in India is at a particularly nascent stage. Be it using whole biomass of filamentous fungi to make alternative meats, tapping into yeast-based flavors, or using mushroom and yeast-derived proteins, application of

Table 13.3 Snapshot of Capital Investment into Fungi-Based ProteinProduction to Date

Year	Investments ($M; USD)	Deal count
2014	$0.1	1
2015	$10.1	3
2016	$11.3	5
2017	$43.2	4
2018	$29.2	13
2019	$100.7	15
2020	$265.9	19
Q1 - Q3 2021	$603.4	12
Total	**$1063.9**	**72**

fungi to create alternative meat, egg, dairy, and seafood products is an emerging field globally. Hence, there are several opportunities for stakeholders across academia, industry, and startups at each step of the value chain to accelerate the broader fungi-based alternative protein field.

With more than six million species of fungi, strain selection has been identified as a crucial step in identifying species by screening through filters such as nutritional value, amino acid profile, RNA content, and toxicity, potential to accumulate heavy metals, allergenicity, organoleptic profile, etc. Many strains isolated from food products are available at culture collection centers like CBS-KNAW culture collection at Westerdijk Fungal Biodiversity Institute, VTT Culture Collection in Finland, and the Microbial Type Culture Collection and Gene Bank (MTCC) in India. These strains can be procured under material transfer agreements for producing various food ingredients. For example, through successful screening of gourmet mushroom species, it is possible to obtain strains for production of mycelium biomass that are particularly advantageous for creating alternative dairy products, due to their umami flavors.

Bringing down the production cost of fungal biomass using liquid-state fermentation is another area where more work is needed. Though filamentous fungi-based alternative meats have existed for decades, price parity with conventional meat products has still yet to be achieved. The cost of end products is not competitive with conventional meat products with key cost barriers including raw material or feedstock costs and the high-capital-expenditure infrastructure required for large-scale production of biomass. The use of side streams from the food industry as feedstock can be instrumental in bringing down end product costs. Simplifying the production systems required for the manufacturing of biomass is another route to bring down initial capital investment.

Current technological advancements in bioreactor design for the pharmaceutical industry could be adapted to meet the requirements for liquid-state fermentation for food applications. Depending on the desired end product and host microorganism, the existing designs and processes can be modified locally to achieve high growth rates, efficient feedstock utilization, and high protein content.

The challenge for food production using microbial hosts is manufacturing target compounds in a cost-effective fashion. Process innovations including the use of perfusion bioreactors have allowed for high productivity in biopharmaceuticals, which can be explored for food applications as well. Also, the quality and purity of target compounds like edible proteins or enzymes need not be as high as desired in pharmaceuticals. Thus, there is potential to lower the relative cost of downstream processing of microbial biomass for food applications.

The manufacturing capacity for fermentation applications in India is currently exhausted because of demands for new vaccines and pharmaceutical products during the global pandemic, but even if spare capacity was available for fermentation, the capacity would need to be adapted for food applications, making it challenging to directly utilize existing capacity for alternative protein applications. Hence, for food applications, new fermentation capacity needs to be established and made accessible to entrepreneurs to exploit fermentation-derived ingredients for the alternative protein sector.

13.13 Safety and Regulatory Considerations

13.13.1 Global

In the United States, the Food and Drug Administration (FDA) provides approvals for ingredients that may be food additives for specific uses as GRAS (generally recognized as safe) substances. Mycoprotein is recognized by the FDA as GRAS, and seven mycoprotein fermentation-enabled products have been launched in the United States under the trade name Quorn [43]. As discussed, other companies using fermentation technology include ENOUGH, The Protein Brewery, and Mycorena.

The following is a list* of food additives derived from fungi that have attained the FDA's GRAS approval and can be referred to during pre-market approval required in India:

- Alpha-galactosidase derived from *Mortierella vinacea* var. *raffinose* utilizer for use in the production of sucrose from sugar beets.
- A solvent extraction process for recovery of citric acid from *Aspergillus niger* fermentation liquor.
- Bakers yeast extract from *Saccharomyces cerevisiae.*
- Bakers yeast glycan from *Saccharomyces cerevisiae.*
- Bakers yeast protein derived from *Saccharomyces cerevisiae.*
- *Candida guilliermondii* as the organism for fermentation production of citric acid.
- *Candida lipolytica* for fermentation production of citric acid.
- Carbohydrase and cellulase derived from *Aspergillus niger* for use in clam and shrimp processing.
- Carbohydrase derived from *Rhizopus oryzae* for use in the production of dextrose from starch.
- Dried yeasts, *Saccharomyces cerevisiae*, *Saccharomyces fragilis*, and dried torula yeast, *Candida utilis.*
- Esterase-lipase derived from *Mucor miehei* var. *Cooney et Emerson* as a flavor enhancer in cheeses, fats and oils, and milk products.
- Fermented microbial protein (FMP) from the fungal microorganism *Fusarium novum yellowstonensis.*
- Flour may contain alpha-amylase obtained from the fungus *Aspergillus oryzae.*
- Gibberellic acid derived by fermentation from *Fusarium moniliforme.*
- Invertase from edible baker's yeast or brewer's yeast (*Saccharomyces cerevisiae*).
- Lactase enzyme preparation from *Kluyveromyces lactis* (previously called *Saccharomyces lactis*) for use in hydrolyzing lactose in milk.
- Milk-clotting enzymes, microbial for use in the production of cheese (milk-clotting enzymes derived from *Endothia parasitica*, *Bacillus cereus*, *Mucor pusillus Lindt*, *Mucor miehei*, and *Aspergillus oryzae* are modified to contain the gene for aspartic proteinase from *Rhizomucor miehei* var. *Cooney et Emerson*).
- Mycoprotein.

- Natamycin derived from *Streptomyces natalensis* and *Streptomyces chattanoogensis.*
- Riboflavin biosynthesized by *Eremothecium ashbyii.*
- *Trichoderma reesi,* used comprehensively in the industry to produce cellulase and other proteins/enzymes for food applications, including non-animal whey protein.
- Vitamin D, produced by ultraviolet irradiation of ergosterol isolated from yeast and related fungi.
- Yeast-malt sprout extract, derived from *Saccharomyces cerevisiae*, *Saccharomyces fragilis*, and *Candida utilis.*

** This list is indicative of ingredients that have received GRAS before November 2021. A consistently updated list is available at GFI-India.org.*

In Europe, the novel food classification is given by the European Food Safety Authority (EFSA) to foods that have not been widely consumed before 1997. Since the regulatory process can be long, many entrepreneurs gather proof that certain fungal strains they are innovating with have been consumed before 1997 to avoid the novel food classification. Within the novel food classification, there is a subcategory of "traditional foods," which are foods consumed outside of the European Union countries and have a history of safe consumption. These traditional foods are regulated slightly differently and are not required to go through rigorous health and safety approvals required for the broader category of novel foods. Fungi-derived food consumed widely outside the European Union can be applied as traditional foods due to the history of safe consumption. Under EFSA, a scientific panel studied the possibility of awarding a "Qualified Presumption of Safety" (QPS) assessment to selected microorganisms and its application in food requests reviewed by EFSA, which would streamline the novel food approval processes. This scientific panel found that for certain species of yeast and filamentous fungi, a blanket approval or QPS status could not be given due to the regular occurrence of infestations and toxins even though the regulator gets regular approval requests for these applications.

13.13.2 Notable Indian Authorities

The Food Safety and Standards Authority of India (FSSAI) is the apex body that controls food safety and standards in India. This is the autonomous regulatory body that takes requests for pre-market review of products that do not have a history of safe consumption in India. The Food Safety and Standards (Food Product Standards and Food Additives) Regulations 2011 has standardized the uses of edible fungi products. These standardized products come under the broad category of "edible fungi products" and have different subcategories with general and specific requirements for each of them.

Fungi in food applications as per FSSAI:

- Freeze-dried fungi
- Fungi grits

- Fungi powder
- Pickled fungi
- Salted fungi
- Fermented fungi
- Fungi in vegetable oils
- Quick-frozen fungi
- Sterilized fungi
- Fungi extract
- Fungi concentrate and dried fungi concentrate

The term "fungi" may be replaced by the terms of the genus or species of the fungi, e.g., "mushroom" or "mushrooms" for the genus *Agaricus*. The method of processing to which the product has been subjected, e.g., "dried," "sterilized," or "quick-frozen," shall be indicated on the label. The regulation has specific sections for the general requirements such as the style (form and presentation of the edible fungi), composition, and specific requirements for each product such as water content, mineral and organic impurities, and permissible limits of damage. The usage of yeast and baker's yeast glycan is regulated and can be used according to good manufacturing practices (GMP) Limits. Baker's yeast glycan is regulated for use as a gelling agent, stabilizer, and thickener in food applications such as bakery products, meat products, seasoning and flavorings, and protein products that are not derived from soy.

Fungal alpha-amylase is listed as an "improver" food additive for bread products with 100 ppm as the limit. There are specific limits placed on the amount of yeast and yeast mold spores that can be found in food products concerning the hygiene and safety of that product. Pimaricin (natamycin) can be used as a preservative in dairy and alternative dairy products up to 40 mg per kg.

13.13.3 Other Authorities

The National Biodiversity Authority (NBA) controls the access to biological resources for commercial utilization. The NBA or the State Authority is established under the Biological Diversity Act 2002. Indian companies interested in commercializing species under the NBA need to get prior permission from the NBA.

13.13.4 Testing and Safety

For food testing and analysis, FSSAI recognizes and notifies National Accreditation Board for Testing and Calibration Laboratories (NABL) accredited food laboratories and also recognizes foreign laboratories to reduce the time in clearance of food consignments at ports.

The FSSAI regulations prescribe that the edible fungi products must be clean, undamaged, and free (as far as possible) of maggot damage and possess the flavor

and taste appropriate to the species. Additionally, there are limits placed on the amount of damage that is permissible in these products. It is stated that Good Agricultural Practices certification and NABL compliance is to be availed to assure compliance with regard to pesticide/chemical residue for the National Horticulture Board.

13.13.5 Import and Export

Most fungi-based proteins are freely exportable unless they fall into the restricted/prohibited list. Under the Agricultural & Processed Food Products Export Development Authority (APEDA), mushrooms are freely exportable.

Food products that are meant for 100% export only must be prepared as per standard/specification and the labeling requirements of the importing country and cannot be sold/supplied for domestic consumption. A No Objection Certificate (NOC) is imperative to export food. Depending on the individual products, other documents may be required.

The following species of edible truffle fungi have been approved for import: *Tuber melanosporum Vittadini*, *Tuber aestivum Vittadini*, *Tuber uncinatum Chatin*, *Tuber mezsentericum Vittadini*, *Tuber magnatum Pico*, *Tuber macrosporum Vittadini*, *Tuber gibbosum Gilkey*, *Tuber borchii Vittadini*, *Tuber brumale Vittadini*, *Tuber indicum*, and *Geopora cooperi*.

13.13.6 Technological Readiness

The Good Food Institute's global scientific team has determined the maturity of each fungal application area based on a literature review of the current state of technology development across the fungal application areas and insights garnered from stakeholder interviews.

The maturity of each application area can be interpreted based on the combination of the technology readiness level and manufacturing readiness level. The technology readiness level ranges from 0, indicating the technology development has not begun, to 5, indicating the presence of product lines in the market by many companies. Similarly, the manufacturing readiness level ranges from 0, indicating the absence of any infrastructure or manufacturing scale facility where the technology relevant to the application area is implemented, to 5, indicating readily available infrastructure or manufacturing facilities where one or multiple technologies relevant to the application area have been scaled in a cost-effective way (Table 13.4). Both the technology readiness and manufacturing readiness levels were determined keeping in mind the Indian as well as the global market (Tables 13.5 and 13.6).

As this analysis demonstrates, based on the developments under various application areas, there has been significant progress in terms of technological readiness for most application areas globally. Manufacturing readiness for most application areas is relatively lower due to high scale-up costs.

Table 13.4 Technology readiness and manufacturing readiness scales

Scale/type of readiness	0	1	2	3	4	5
Technology readiness level	No academic research	Only academic research	Proof of concept validated at lab scale / experimental prototypes / technologies reported by companies / startups	Prototypes / technology demonstrated to be economically viable	Few companies have established product lines for this application area	Many companies have established product lines for this application area
Manufacturing readiness level	No existing infrastructure capacity to support this activity	No infrastructure capacity to support this activity but infrastructure available for other applications	Infrastructure established by few companies, but cost is prohibitive, no scalable solutions exist	Infrastructure established by few companies, but cost is prohibitive, scalable solutions demonstrated to bring down costs	Infrastructure established by few companies and cost is no longer prohibitive	Infrastructure is commonplace, high scalability of production and processing techniques

Table 13.5 Maturity ratings for application area globally

	Whole-cut meats	Textured meats	Protein isolates	Flavor compounds	Scaffolding materials	Fermented protein
Technology readiness level	3	5	3	5	3	5
Manufacturing readiness level	4	3	2	5	0	4

Table 13.6 Maturity ratings for application areas in India

	Whole-cut meats	Textured meats	Protein isolates	Flavor compounds	Scaffolding materials	Fermented protein
Technology readiness level	0	3	2	5	2	0
Manufacturing readiness level	1	1	1	5	0	1

In India particularly, both technology and manufacturing readiness levels are lower than global readiness levels. This can partly be explained by the absence of indigenous companies that are exploring novel food applications from fungi.

13.14 Conclusions

As the world slowly emerges from a traumatic period of pandemic-driven supply chain disruptions and public health emergencies, forward-thinking governments and business leaders are motivated to construct a more resilient global food system that could prevent future crises before they emerge. Fungi-based alternative proteins are an essential element of that shift toward increased food security and sustainability.

Every aspect of the food sector, from academic researchers and policymakers to investors and entrepreneurs, has a role to play in breaking through current bottlenecks and fully exploring the potential of the fungi kingdom. Conventional meat, dairy, and egg producers have been scaling up manufacturing and driving down costs for decades, but we do not have the luxury of such an expansive timeline to elevate alternative proteins. Whether the global food system is ready or not, protein demand is surging, and the Asian continent is at the biggest risk of falling short.

Embedded in that steep climb is a mountain of lucrative opportunities for those scientists and technologists astute enough to seize them. The Good Food Institute and other cross-sector alternative protein think tanks stand ready to work with stakeholders of all stripes to accelerate innovation beyond current thresholds and help humanity meet this moment.

References

1. Lieberg A, Sin H (2021) The need for change, The good food institute Asia Pacific. Accessed 29 November 2021. https://drive.google.com/file/d/1OZ3-dVgheE671p73HRVRhhxGR36BWKWi/view?usp=sharing
2. Huling R, Siu E (2020) Asian Cropportunities. In: The good food institute Asia Pacific. Accessed 29 November 2021. https://www.gfi-apac.org/resources/asian-cropportunities/
3. Climate Advisers and The Good Food Institute (2021) Global food system transition is necessary to keep warming below 1.5°C, Accessed 29 November 2021. https://gfi.org/wp-content/uploads/2021/04/FINAL_Climate-Advisers_Good-Food-Institute-Alternative-Proteins-Report.pdf
4. Moore D, Chiu SW (2001) Fungal products as food. Eds. Pointing, SB and Hyde, KD in bio-exploitation of filamentous fungi. Fungal Divers Res Series 6:223–251
5. El Youssef C, Bonnarme P et al (2020) Sensory improvement of a pea protein-based product using microbial co-cultures of lactic acid bacteria and yeasts. Foods 9:349. https://doi.org/10.3390/foods9030349
6. Sutton M, Barr S (2018) Food biotechnology. Scientific e-Resources
7. Wiebe M (2002) Myco-protein from *Fusarium venenatum*: a well-established product for human consumption. Appl Microbiol Biotechnol 58(4):421–427
8. Knight N, Roberts G et al (2001) The thermal stability of Quorn™ pieces. Int J Food Sci Technol 36:47–52. https://doi.org/10.1046/j.1365-2621.2001.00424.x
9. Bomkamp C, Skaalure S et al (2021) Scaffolding biomaterials for 3D cultivated meat: prospects and challenges. Adv Sci 2021:2102908
10. Rodríguez-Vázquez M, Vega-Ruiz B et al (2015) Chitosan and its potential use as a scaffold for tissue engineering in regenerative medicine. BioMed Res Int 2015
11. Berthod F, Germain L et al (2006) Extracellular matrix deposition by fibroblasts is necessary to promote capillary-like tube formation in vitro. J Cell Physiol 207(2):491–498

12. Bitar KN, Zakhem E (2014) Design strategies of biodegradable scaffolds for tissue regeneration. Biomed Eng Comp Biol, 6, pp. BECB-S10961
13. Ren X, Chen C et al (2018) Biodegradable chitosan-based composites with dual functions acting as the bone scaffold and the inflammation inhibitor in the treatment of bone defects. Int J Polym Mater Polym Biomater 67(12):703–710
14. Croisier F, Jérôme C (2013) Chitosan-based biomaterials for tissue engineering. Eur Polym J 49(4):780–792
15. Rubio N, Datar I et al (2019) Cell-based fish: a novel approach to seafood production and an opportunity for cellular agriculture. Front Sustain Food Syst 3:43
16. Nitschke J, Altenbach HJ et al (2011) A new method for the quantification of chitin and chitosan in edible mushrooms. Carbohydr Res 346(11):1307–1310
17. Pochanavanich P, Suntornsuk W (2002) Fungal chitosan production and its characterization. Lett Appl Microbiol 35(1):17–21
18. Wu T, Zivanovic S et al (2004) Chitin and chitosan value-added products from mushroom waste. J Agric Food Chem 52(26):7905–7910
19. Campuzano S, Pelling AE (2019) Scaffolds for 3D cell culture and cellular agriculture applications derived from non-animal sources. Front Sustain Food Syst 3:38
20. Nasseri AT, Rasoul-Amini S et al (2011) Single cell protein: production and process. Am J Food Technol 6(2):103–116
21. Longvah T, Deosthale YG (1998) Compositional and nutritional studies on edible wild mushroom from Northeast India. Food Chem 63(3):331–334
22. Chang S-T (2008) Overview of mushroom cultivation and utilization as functional foods. In: Cheung PCK (ed) Mushrooms as functional foods. https://doi.org/10.1002/9780470367285.ch1
23. Mau JL, Chao GR et al (2001) Antioxidant properties of methanolic extracts from several ear mushrooms. J Agric Food Chem 49(11):5461–5467
24. Mattila P, Könkö K et al (2001) Contents of vitamins, mineral elements, and some phenolic compounds in cultivated mushrooms. J Agric Food Chem 49(5):2343–2348
25. Edwards DG, Cummings JH (2010) The protein quality of mycoprotein. Proc Nutr Soc 69 (OCE4)
26. Fairweather-Tait SJ, Southon S (2003) Bioavailability of nutrients. In: Encyclopedia of food sciences and nutrition, 2nd edn, pp 478–484
27. Bottin JH, Jonathan RS et al (2016) Mycoprotein reduces energy intake and postprandial insulin release without altering glucagon-like peptide-1 and peptide tyrosine-tyrosine concentrations in healthy overweight and obese adults: a randomised-controlled trial. Br J Nutr 116(2):360–374
28. Müller H, Lindman AS et al (2003) The serum LDL/HDL cholesterol ratio is influenced more favorably by exchanging saturated with unsaturated fat than by reducing saturated fat in the diet of women. J Nutr 133(1):78–83
29. Turnbull WH, Ward T (1995) Mycoprotein reduces glycemia and insulinemia when taken with an oral-glucose-tolerance test. Am J Clin Nutr 61(1):135–140
30. Turnbull WH, Leeds AR et al (1990) Effect of mycoprotein on blood lipids. Am J Clin Nutr 52(4):646–650
31. Turnbull WH, Leeds AR et al (1992) Mycoprotein reduces blood lipids in free-living subjects. Am J Clin Nutr 55(2):415–419
32. Turnbull WH, Walton J et al (1993) Acute effects of mycoprotein on subsequent energy intake and appetite variables. Am J Clin Nutr 58(4):507–512
33. Nielson IQ (2019) The F Word: Flexitarian is not a curse to the meat industry. Accessed 29 November 2021. https://nielseniq.com/global/en/insights/analysis/2019/the-f-word-flexitarian-is-not-a-curse-to-the-meat-industry/
34. Mintel (2020) Plant-based proteins: incl impact of COVID-19. Accessed 29 November 2021, https://reports.mintel.com/display/986972/
35. Turnwald B, Boles D et al (2017) Association Between Indulgent Descriptions and Vegetable Consumption: Twisted Carrots and Dynamite Beets. JAMA Internal Medicine

36. Parry J, Szejda K (2019) How to drive plant-based food purchasing: key findings from a Mindlab study into implicit perceptions of the plant-based category. Research Report. The Good Food Institute, Washington, DC. Available at https://go.gfi.org/Mindlab-Strategic-Recommendations
37. Frothingham S (2019) What is Mycoprotein and is it safe to consume?, Healthline, Accessed 29 November 2021. https://www.healthline.com/health/mycoprotein
38. Roerink, A., 2019, *The power of meat 2019*, 210analytics, accessed 29 November 2021, https://www.fmi.org/docs/default-source/webinars/power_of_meat_2019_final_webinar.pdf?sfvrsn
39. Askew K (2021) Unilever partners with mycoprotein supplier ENOUGH on 'game changing' protein innovation, FoodNavigator, Accessed 29 November 2021
40. Ritchie H, Laird J et al (2017) 3f bio: halving the cost of mycoprotein through integrated fermentation processes. Ind Biotechnol 13(1):29–31
41. Mycorena (2021) Mycorena cracking the code of the world's first fungi-based fat. Accessed 29 November 2021. https://mycorena.com/mycotalks/mycorena-cracking-the-code-of-the-worlds-first-fungi-based-fat-ingredient
42. Poinski M (2021) *Hormel partners with the better meat co. to develop meat analog products*, food dive, Accessed 29 November 2021. https://www.fooddive.com/news/hormel-partners-with-the-better-meat-co-to-develop-meat-analog-products/607653/
43. Denny A, Aisbitt B et al (2008) Mycoprotein and health. Nutr Bull 33(4):298–310

Part IV

Fungal Enzymes and Other Biotech Products

Fungal Enzymes in the Production of Biofuels

14

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Abstract

Due to the exhaustion of non-renewable fossil fuel resources, rising costs of petroleum-based fuels, efforts to save energy, and the acceleration of global warming, renewable energy has received attention on a global scale. Biofuels have become a viable alternative energy source to cut greenhouse gas emissions and combat global warming. It's also been demonstrated to be a means of achieving long-term energy sustainability. There are four generations of biofuels based on feedstocks. Before fermentation, edible feedstocks like as sugarcane juice, cereals, and starch-rich root tubers are hydrolysed with the aid of amylases and inulinases to produce first-generation biofuels. Since they compete with human food, although making up the majority of the biofuel produced at the time, these are being opposed by a variety of organizations. The second-generation biofuels are created to overcome these challenges by utilizing non-edible biomass, such as agricultural waste residues, agro-industrial waste, horticultural wastes, and food wastes rich in cellulose, hemicellulose, pectin, and starch saturated with refractory lignin. The carbohydrates in these feedstocks are hydrolysed enzymatically utilizing cellulases, hemicellulases, pectinases, and amylases after they have been delignified through physio-chemical or biological pretreatments employing ligninases. Third-generation biofuels are created from starch- and cellulose-rich algal biomass using amylase- and cellulase-based hydrolysis. Third-generation biofuels use third-generation hydrolytic enzymes along with genetic engineering and molecular biology to enhance algal strains. Fourth-generation biofuels are an improvement over third-generation biofuels. The environmentally friendly generation of biofuels relies heavily on enzymes.

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Fungal enzymes are extracellular and exhibit substantially better productivity with simple downstream processes than bacterial enzymes, which are largely intracellular or spore-bound and have low yields. Biotechnological innovations gave rise to strain enhancement, which increased the productivity of certain hydrolytic enzymes and their adaptability to diverse environmental conditions during the conversion of various feedstocks to biofuel. Different generations of biofuels, the enzymes utilized in their production, the significance of each generation, and the potential of biofuels as a source of sustainable energy are all covered in this chapter.

Keywords

Biofuels · Fungal enzymes · Laccases · Cellulases · Hemicellulases · Amylases · Pectinase

14.1 Introduction

Energy is the backbone of societal structure and is divided into two categories, non-renewable energy and renewable energy [1]. The increasing human population and industrialization are directly proportional to the demand for non-renewable energy resources [2]. Experts estimate that conventional fossil fuels account for 87% of the world's energy consumption [1]. As a result of the high demand, non-renewable energy resources are gradually depleting. The industrial, transportation, and electrical sectors are mostly to blame for energy consumption, which has a negative impact on the climate and causes glaciers to melt, sea levels to rise, and a rapid loss of biodiversity in addition to greenhouse gas emissions [3]. The instant must seek out alternative, sustainable, and financially viable fuel sources with low GHG emissions. Renewable energy sources are reportedly divided into two categories: clean energy and bioenergy. Clean energy comes from ocean water waves, air currents, hydropower, and solar sources, whereas bioenergy comes from organic matter via biochemical processes. Alcohols, biodiesel, biogas, hydrogen, and other bioenergy resources are examples of biofuels [4]. Biofuels have been extensively examined and are deemed credible and cost-effective energy sources [5]. The conversion of lignocellulosic biomass by biological means seems more promising [6]. Technology development has significantly turned toward effective and unconventional biomass feedstocks as a result of the expanding worldwide demand for biofuels in recent decades. According to study articles produced over the past 20 years worldwide, especially from Asia, Europe, and the United States, major research attention has been made to practical and affordable biofuel resources due to the rising global biofuel consumption pattern [7].

Primary and secondary biofuels are categorized as being produced either directly or indirectly from energy. Wood chips, forest litter, dried animal dung used for cooking, and fuel woods are examples of primary fuels that are unprocessed biofuels. Before fossil fuels were discovered, these fundamental biofuels served as

the main source of energy. Primary biofuels are still the major source of energy in rural developing nations, mostly for cooking and heating, even if they are no longer recognized for everyday usage in metropolitan areas due to environmental sustainability. There are two types of secondary biofuels: conventional biofuels and advanced biofuels. These secondary biofuels are created by modifying primary biofuels that are processed chemically or biologically. Conventional or first-generation biofuels are made from damaged or edible crops that are starchy or sugary [8]. The process involved in the generation of first-generation biofuels generally involves substrate dilution or hydrolysis by enzymes and fermentation. Advanced biofuels, also known as non-edible organic matter residual fuels, are second-generation fuels, and their production needs to pretreat the feedstocks physically, chemically, or enzymatically before enzyme-mediated or acid hydrolysis and, ultimately, fermentation. Hydrolysis and fermentation are also used to create third-generation fuels from seaweeds and algae. Engineered photosynthetic microorganisms and artificial photosynthetic devices that employ synthetic biology methods to manufacture electro fuels and photobiological solar fuels by directly converting solar energy into fuels are considered fourth-generation biofuels [9]. Genetic engineering is used to upgrade third-generation biofuels to create fourth-generation biofuels.

14.2 First-Generation Biofuels

Starch and sugar-based feedstocks are the two categories of edible feedstocks used to make first-generation biofuels [10]. The starch-based feedstock includes things like potatoes, barley, corn, and wheat. However, sugarcane and sugar beet are examples of sugar-based feedstocks. The feedstocks are grown in monocultures, in which only one kind of feedstock is present at any one moment in the same location. Gelatinization, which breaks down starch's crystallinity, is followed by enzymatic hydrolysis for the production of monomeric sugar and fermentation in the production of starch-based biofuels. Sugar-based feedstocks, such as sugar cane juice, are fermented directly or require only dilution before fermentation, as in the case of molasses. The world is interested in the development of first-generation biofuels made from edible biomasses like wheat, rice, potatoes, sugarcane, barley, and vegetable oil. Using fermentation and transesterification, sugars and oils are converted from organic components into bioethanol or biodiesel, respectively. While rapeseed, soybeans, and sunflower are said to be good sources of feedstock for the manufacture of biodiesel, Kopp et al. [11] claimed that sugar-based crops like sugar beet and sugarcane contain a significant quantity of saccharose that may be extracted and fermented into bioethanol. Enzymes are crucial for the effective and sustainable conversion of feedstocks. The creation of food fibre for human use, which can undermine environmental sustainability and economic viability, has been a problem for first-generation biofuels [12]. Second-generation biofuels were created as a result of growing concern about the shortcomings of first-generation biofuels.

14.2.1 Enzymes for the Production of First-Generation Biofuels

Enzymes are mostly utilized at the hydrolysis step in the synthesis of first-generation biofuels. Amorphous starch is hydrolysed to glucose by amylases, which is then fermented by yeast to ethanol, and then distilled to produce bioethanol from grains. One key factor affecting the effectiveness of the process and ethanol generation is the efficient conversion of starch into glucose. Overcoming the detrimental impacts of non-starch grain components, such as protein, fat, pentosan, and β-glucan, is one of the difficulties in attaining effective starch hydrolysis. Enzymes like pullulanase and inulinase, coupled with conventional amylases for starch hydrolysis, can be added to reverse these detrimental effects. These components can be hydrolysed into monomer units, which are subsequently fermented to create ethanol. These hydrolytic enzymes may originate in microorganisms, animals, or plants. It is theoretically possible to obtain such an enzyme from microbes with properties and potentials that are equivalent to those of both animal and plant enzymes.

14.2.1.1 Amylases

First-generation biofuels require a lot of starch as one of their main substrates. Therefore, the enzymatic hydrolysis of starch substrates is a preferable technique since it prevents inhibitor buildup. Furthermore, the enzymes contain no secondary reactions and work in a highly precise manner. Glucose units in starch are connected by glycosidic linkages. Figure 14.1 shows the architecture of the two types of polymeric units that make it up: amylose and amylopectin. By α-1,4 glycosidic linkages, amylose is made up of linearly connected glucose units. Amylopectin is made up of linear glucose chains with an α-1,4 linkage that is joined to the side chains by an α-1,6 linkage [13].

Amylases, also known as glycosyl hydrolases (GH), are classified by the International Union of Biochemistry and Molecular Biology (IUBMB) into three groups: endo-amylases, exo-amylases, and debranching enzymes. The modes of action of each of these enzymes on starch are shown in Fig. 14.2. The numerous kinds of starch-degrading enzymes include the following:

1. **Endo-amylases or α-amylases (EC 3.2.1.1):** It produces oligosaccharides and dextrins by cleaving the α-1,4-bonds found in the interior regions of amylase and amylopectin, causing the solution's viscosity to decrease.
2. **Exo-amylase or β-amylases (EC 3.2.1.2):** In order to release limit dextrins and β-maltose, it only dissolves α-1,4-bonds that are present at the non-reducing ends.
3. **γ-Amylase or amyloglucosidase or glucoamylase (EC 3.2.1.3):** It also functions as a debranching enzyme, cleaving the last α-1,4 links at the non-reducing end of amylase and amylopectin to produce glucose.
4. **Pullulanase (EC 3.2.1.41):** Pullulanase (EC 3.2.1.41), also known as dextrin 6-glucanohydrolase, pullulan 6-glucanohydrolase, limit dextrinase, and amylopectin 6-glucanohydrolase, is present in a variety of fungal species. Pullulanase from microorganisms is becoming more and more well-liked due to its clarity on α-1, 6 connections in pullulan (a linear glucan mostly composed of maltotriosyl

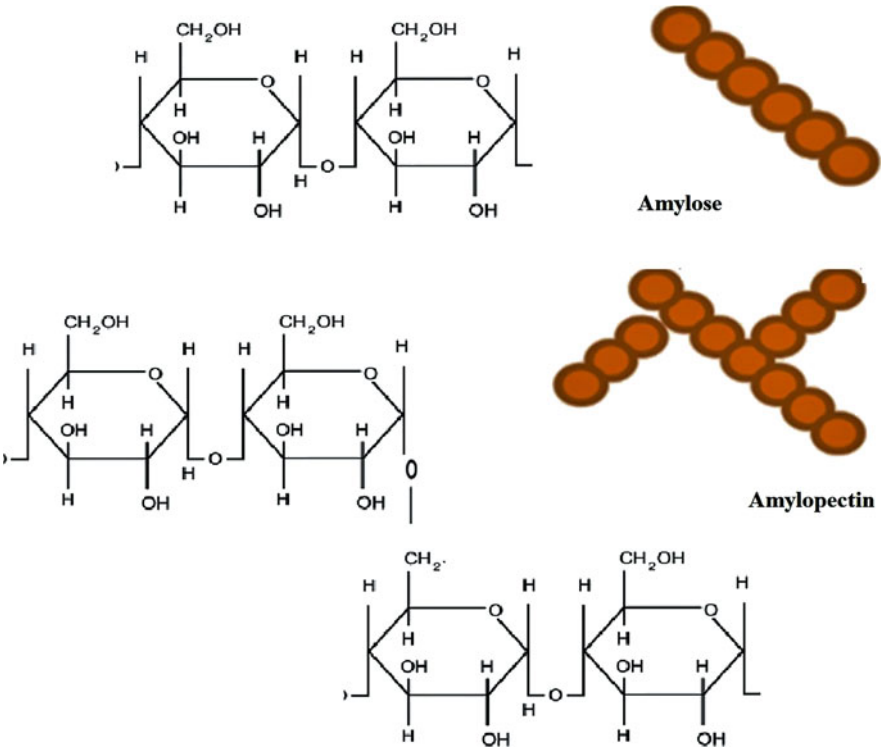

Fig. 14.1 Structure of starch, the substrate for amylases [14]

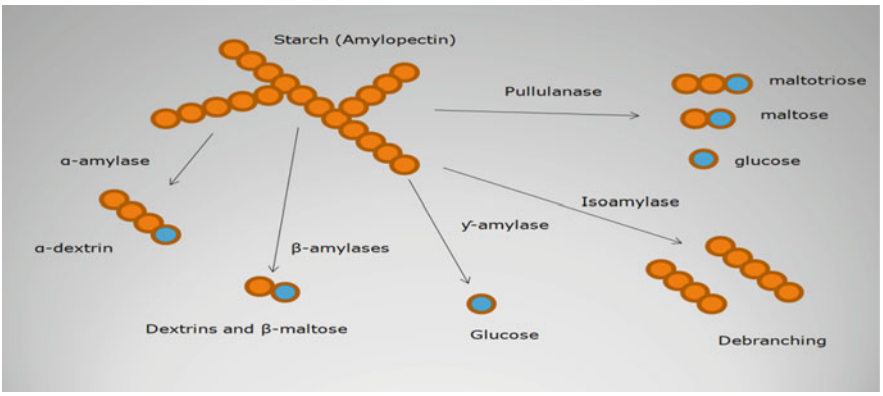

Fig. 14.2 Mode of action of amylases [23]

units connected by 1, 6-α-bonds). Based on differences in substrate types and reaction outcomes, pullulan hydrolytic enzymes are categorized into three groups. Pullulanase type I enzymes, which may also hydrolyse the α-(1,6) glucosidic linkage in pullulan and branched polysaccharides, have received the majority of attention [15–19]. The exact debranching ability of pullulanases type II, also known as amylopullulanases, to hydrolyse either α-(1,6) or α-(1,4) glucosidic links makes them crucial in the starch processing business. This enzyme targets the α-(1,4) bonds in starch, amylose, and amylopectin and debranches pullulan, producing maltotriose as a by-product [18, 20]. Maltotriose is produced when pullulanases I and II cleave the α-(1,6) glucosidic links in pullulan [21]. It has been revealed that the pullulanase type III enzyme breaks down the α-(1,6) and α-(1,4)-glucosidic linkages in pullulan to create a combination of maltotriose, panose, maltose, and glucose. Maltotriose and maltose can also be produced by this enzyme by solubilizing starch, amylose, and amylopectin.

5. **Isoamylase (EC 3.2.1.68):** Only one enzyme, isoamylase (EC 3.2.1.68), also known as glycogen 6-glucanohydrolase, is capable of entirely debranching starch or glycogen [22].

Numerous fungi have been reported to produce amylases. Several species of the genera *Aspergillus* and *Penicillium* are effective fungal amylase producers. The list of several fungi known to generate amylases is shown in Table 14.1.

14.2.1.2 Inulinases

Inulin hydrolysis has been associated with endo- and exo-inulinases (Table 14.2; Fig. 14.3). Exo-inulinase lowers the terminal fructose residues of inulin from the non-reducing end, whereas endo-inulinase operates on the internal linkages of the inulin molecule but lacks invertase activity [47, 48].

Table 14.1 List of various fungi producing amylases

Fungal strain	Reference
Aspergillus awamori	[24]
Aspergillus flavus	[25, 26]
Aspergillus fumigatus	[27, 28]
Aspergillus niger	[29–35]
Aspergillus oryzae	[36, 37]
Aspergillus tamarii	[38, 39]
Penicillium camemberti	[40]
Penicillium citrinum	[41]
Penicillium expansum	[42]
Penicillium janthinellum	[43]
Penicillium notatum	[42]
Penicillium oxalicum	[44]
Trichoderma harzianum	[45]
Trichoderma pseudokoningii	[46]

Table 14.2 Various components of inulinases and their mode of action

Polymer	Nature of polysaccharide	Glycosidic bond present	Enzymes required	Mode of action	Hydrolytic product
Inulin	Homo polysaccharide	β-(2 → 1) fructosyl linkage	Endo-inulinase (EC 3.2.1.7)	That splits down inulin into inulooligosaccharides	Inulooligosaccharides
			Exo-inulinase (EC 3.8.1.80)	Breaks inulin's terminal fructose units	Fructose

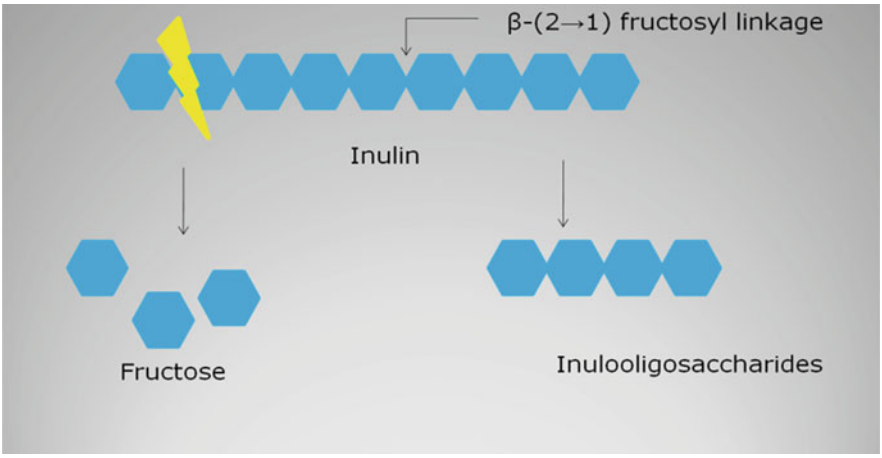

Fig. 14.3 Microbial exo- and endo-inulinase enzymes are depicted schematically. Endo-inulinase generates inulooligosaccharides, whereas exo-inulinase releases fructose from the macromolecule [49]

14.3 Second-Generation Biofuels

Second-generation biofuels are typically produced from non-edible lignocellulosic biomass, such as plants or even whole crops; organic municipal solid wastes; by-products like sugarcane bagasse, cereal straw, and wood waste; as well as feedstock like short-rotation forests, vegetative grasses, and additional energy crops [50].

As a substitute for first-generation lignocellulosic feedstock in the manufacture of biofuels and chemicals, we have been obliged to switch to second-generation lignocellulosic feedstock due to the controversy surrounding these feedstocks and food security [51]. Dry agricultural wastes, by-products of advanced manufacturing processes, and forest leftovers, also known as lignocellulose, are all used as fuel for the combustion process. The anticipated yearly output of lignocellulosic materials from agricultural production and forest destruction is between five and eight million tonnes, which is less than the annual use of conventional crude oil [52]. Lignocellulose-based processes have the potential to cut GHG emissions, which benefits the economy and adds to energy security given the long-term application of chemical and biofuel production. The biochemical, hybrid, and thermochemical pathways for biomass conversion to biofuels are examples of technological advancements in this field. Thermochemical processing uses gasification and pyrolysis to turn lignocellulose into synthesis gas (carbon monoxide and hydrogen). The pretreatment step in the process of turning lignocellulosic wastes into liquid fuel, namely, acetone, butanol, and ethanol (ABE) fermentation, involves saccharifying the pretreated substrate by hydrolysis, which releases monomeric sugars. The process of turning

these monomeric sugars into either ABE or ethanol using the appropriate microorganism is the final step. These sustainable lignocellulosic biomasses are currently being used as a source for the commercial generation of biofuels through chemical methods. In order to reduce the number of harsh chemicals required and to allow for the inclusion of certain wastewater treatments, the emphasis has changed to incorporate enzymes for each phase. The discovery and development of certain enzyme systems for the pretreatment and hydrolysis processes are the results of research. However, because they are more expensive and have lower efficacy than chemical therapies, enzymes are not extensively adopted. Researchers have focused their efforts primarily on these significant cost-increasing issues, and advancements are achieved for effective and affordable enzyme manufacturing.

14.3.1 Enzymes in the Pretreatment of Lignocellulosic Residues for the Production of Second-Generation Biofuels

The first set of enzymes believed to be engaged in the conversion of lignocellulosic residues occurs in the pretreatment stage. In order to facilitate the hydrolysis of cellulose and hemicellulose, the primary goal of pretreatment is to delignify the refractory lignin content. To enable the hydrolysis of cellulose, the second objective is to decrease its crystallinity. The secret to designing a bioethanol production system that is both efficient and affordable is to pinpoint particular, highly effective enzyme systems, enzyme complexes, or enzymatic cocktails that target and enhance the destruction of the complex lignin-hemicellulose in biomass. For biological processing of lignocellulosic residue, they are used. Their tailored action strategy prevents the production of fermentation inhibitors, making them the most economical, efficient, and ecologically benign alternative to harsh chemical treatments. Hemicellulases and ligninases are two examples of ligno-hemicellulolytic enzymes. These unique enzymes target just certain lignin and hemicellulose, leaving cellulose alone, which is needed in its natural state for the manufacture of biofuel. The distinctive feature of enzyme-assisted delignification is that it preferentially targets lignin molecules, generating phenolic intermediates that act as naturally occurring ligninase mediators, allowing some of the other non-phenolic lignin molecules to be oxidized. A unique enzyme called ligninase causes the lignin to be disrupted. Such white-rot fungus generates a variety of lignin-degrading enzymes, including lignin peroxidase, laccase, mannanase peroxidases, and manganese-dependent peroxidases. The most widely utilized, studied, and investigated lignin-disrupting enzyme is laccase [53].

14.3.1.1 Laccases

White-rot fungi, which are members of the basidiomycetes and have highly developed ligninolytic systems composed mostly of lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13), and laccase, are the main microorganisms in nature that cause delignification (EC 1.10.3.1) [53, 54]. A long-chain, highly branching heterogeneous block known as lignin is largely cross-linked with

hemicellulose via both covalent and hydrogen bonds [55]. Lignin is broken down into CO_2 and water by lignin-degrading enzymes produced by the white-rot fungus. One of the most used and commercially valuable enzymes is laccase. Laccases are copper-based phenoloxidases that catalyse the oxidation of anilines, aromatic thiols, and phenols found in lignin. Because microbes can quickly take them as carbon sources, the removal of phenols from lignin by laccase encourages the development of microorganisms by reducing the log phase. With the aid of mediators, laccase may directly attack the nonphenolic and phenolic lignin subunits, changing the structure of lignocellulosic biomass [56].

As an oxidoreductase, laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) may speed up the oxidation of ortho- and para-diphenols, aminophenols, polyphenols, polyamines, lignin, and aryl diamines as well as a few inorganic salts. It can also reduce O_2 to H_2O [53, 57, 58]. A dimeric or tetrameric glycoprotein called laccase has four copper atoms that help form the active site [56, 59–61]. The area of type I copper is where the substrate binds and electronic oxidation occurs. The copper-cysteine interactions at this location are very normal covalent connections, and these are what give the enzyme its blue hue. The reduction of O_2 to H_2O happens as a result of this electron being transferred to a trinuclear cluster made up of two type II and one type III copper atom in Fig. 14.4. Four hydrogen-donating substrates are first oxidized by a single electron by laccase, which then catalyses the four-electron reduction of oxygen to water [56, 60, 62]. The reactive radical generated can undergo non-enzymatic processes such as (1) covalent pairing to create dimers, oligomers, and polymers through C-C, C-O, and C-N links [63], (2) releasing monomers by rupturing the covalent bonds of bigger molecules like lignin [64], and (3) targeting the aromatic ring [63].

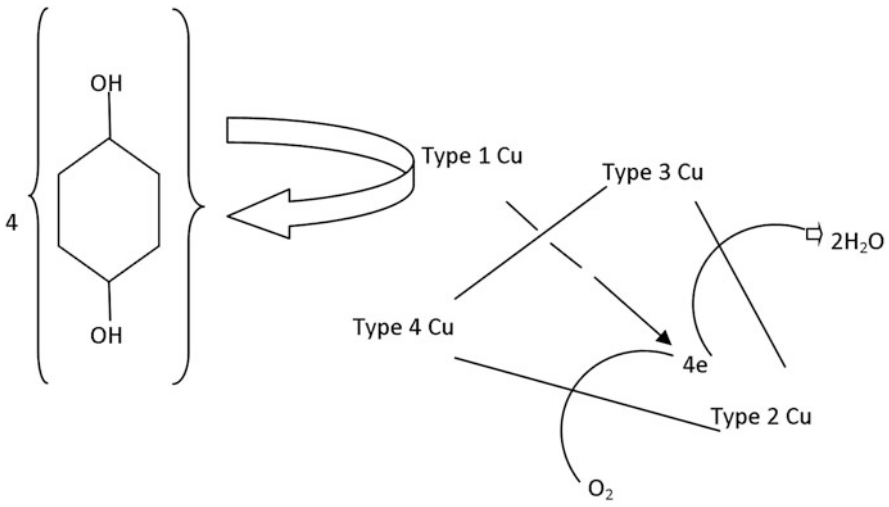

Fig. 14.4 Laccase's generalized way of activity on substrates [99]

Table 14.3 Fungi involved in laccase production

Fungal strain	Reference
Aspergillus fumigates	[69]
Deuteromycete pestalotiopsis	[70]
Echinodontium taxodii	[71]
Galactomyces geotrichum	[72]
Phlebia radiata	[68]
Pleurotus ostreatus	[66, 73]
Pleurotus sajor-caju	[74]
Pseudolagarobasidium acaciicola	[75]
Pycnoporus sanguineus	[76]
Pyrenophora phaeocomes	[77]
Schizophyllum commune	[65]
Trametes	[78]
Trametes versicolor	[79]
Trichoderma viride	[80]

There are already more than 100 laccase enzymes that have been successfully extracted, purified, and identified. *Monocillium indicum* laccase was the very first laccase to be recognized as possessing peroxidative activity. White-rot fungus like *Schizophyllum commune* are among the lignolytic microorganisms [65], *Trametes versicolor* (Iandolo et al., 2011), *Pleurotus ostreatus* [66], *Cerrena consors* [67], *Phlebia radiata* [68], etc. In comparison to bacterial laccase, which is mostly intracellular or spore-bound and has low yields, fungal laccase has a significant benefit since it is extracellular and secretes a lot more in the environment. The recovery of fungal enzymes is also less complicated than that of bacterial enzymes. Table 14.3 shows the fungi that are involved in laccase production. The biomass from the processed lignocellulosic residues is hydrolysed and converted to monomeric sugars using saccharification. Enzymatic hydrolysis or acid hydrolysis both work to saccharify substances.

14.3.2 Acid Hydrolysis of Pretreated Residues

Since a very long time ago, diverse substrates have been hydrolysed using acids. The two most often used acids are H_2SO_4 and HCl, which may be utilized in both diluted and concentrated forms and at varying concentrations [81]. Dilute acid hydrolysis involves two processes. The first step in the process is the saccharification of carbohydrates, and if the reaction persists, sugars will then be converted to furfurals. Because cellulose breaks down more slowly than hemicellulose, a two-stage process is necessary to prevent the formation of furfurals from the sugars released from hemicellulose. The first stage of the process recovers the sugars from the hemicellulose under mild conditions, and the second stage recovers the sugars from the cellulose under harsher conditions. Strong acids like sulphuric acid, hydrochloric acid, nitric acid, or phosphoric acid saccharify the cellulose and hemicellulose of

lignocellulosic biomass at a suitable temperature for hydrolysis by strong acids. To prevent the dilution of acid during the hydrolysis process, biomass is first dried to achieve a moisture content below 10%. Hemicellulose is hydrolysed with strong acids more so than cellulose. As a result, the hemicellulose-derived monomeric sugars are exposed to acid for an extended period, which leads to their breakdown. It takes a lot of energy and moves slowly. Chemical recovery is necessary owing to both economic and environmental considerations.

14.3.3 Enzymatic Hydrolysis of Pretreated Residues

Because it does not produce inhibitors, enzymatic hydrolysis of the pretreated substrate is preferred over acid hydrolysis. Furthermore, there are no secondary processes involved in the activities of the enzymes, which are quite specialized. The cellulose and hemicellulose's crystalline structure may now be attacked by enzymes, releasing sugars, thanks to the pretreatment of the substrate (Fig. 14.5). Cellulases and hemicellulases are required to break down cellulose and hemicellulose since

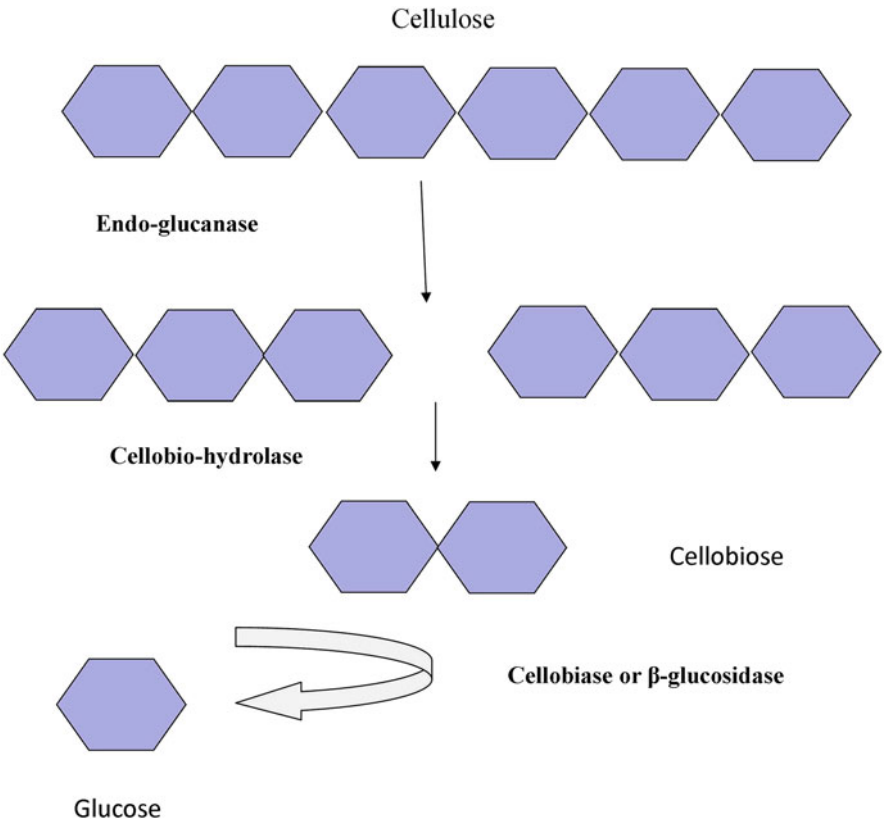

Fig. 14.5 The mode of action of the enzymes implicated in cellulose degradation [84]

they are the two main carbohydrates present in the cell wall structure [82]. The pretreated substrate must also include starch and pectin in order for amylases and pectinases, the corresponding enzymes, to fully saccharify the substrate.

14.3.3.1 Enzymes Used in the Hydrolysis of Pretreated Lignocellulosic Biomass to Produce Second-Generation Biofuels

Several cellulolytic, hemicellulolytic, and pectinolytic enzymes are engaged in their hydrolysis before fermentation since the substrates used for the synthesis of second-generation biofuels mostly comprise cellulose, hemicellulose, and pectin that are left over after the pretreatment stage.

14.3.3.1.1 Cellulases

In reality, lignocellulose depends on the collaboration of several enzymes, the most significant of which are cellulases. Since cellulases hydrolyse cellulose's β-1,4-D-glucan linkages to produce cellobiose and glucose, they are glycosyl hydrolases. In order to enhance parallel orientation and create a crystalline structure with an extended flat twofold helical shape that restricts enzyme accessibility, hydrogen bonds and van der Waal forces are used to join nearby cellulose molecules [83]. Three enzymes must work together in order to completely break down the cellulose structure (Fig. 14.5):

1. **Endo-glucanase (EC 3.2.1.4):** By creating random intrinsic breaks in the amorphous portion of cellulose, it produces short-chain oligomers with non-reducing or reducing ends.
2. **Cellobio-hydrolase (EC 3.2.1.91):** Cellobiose, the repeating unit of two glucose molecules, is created by hydrolysing the non-reducing ends created by the enzymatic activity of endoglucanase.
3. **Cellobiase or β-glucosidase (BG) (EC 3.2.1.21):** To create monomeric glucose units, it disassembles cellobiose units.

Fungi are regarded as potential cellulase producers and microorganisms as potent cellulase producers. Additionally, soft-rot fungi, white-rot fungi, and brown-rot fungi are also cellulase producers [82, 85, 86]. Due to their greater penetrating capabilities and wide range of substrate usage, fungi are always preferred over bacteria. Microorganisms that produce cellulase may thrive on various carbohydrates but prefer to grow on cellulose. The several fungi that produce cellulase are shown in Table 14.4.

14.3.3.1.2 Hemicellulases

Hemicellulose, which is made up of xylan, mannan, arabinan, and galactan, is the second most abundant polymer in nature. It is soluble in aqueous alkali but not in water or any chelating agent [113]. The enzymatic sector for hemicellulases is continuously growing despite the fact that hemicellulases are utilized in several industrial operations. They provide greater opportunities for enantioselective chemical and enzymatic modifications than cellulose due to the range of sugar

Table 14.4 List of various fungi producing cellulases

Fungal strain	Reference
Aspergillus flavus	[87–89]
Aspergillus fumigatus	[90, 91]
Aspergillus niger	[30–33, 85, 86, 88, 92]
Aspergillus oryzae	[93]
Fusarium graminearum	[15]
Fusarium oxysporum	[94]
Fusarium solani	[95, 96]
Penicillium brevicompactum	[97]
Penicillium citrinum	[98]
Penicillium decumbans	[99]
Penicillium echinulatum	[100]
Penicillium funiculosum	[101]
Penicillium janthinellum	[43]
Penicillium oxalicum	[102–104]
Penicillium sclerotiorum	[105]
Trichoderma harzianum	[45, 106–108]
Trichoderma longibrachiatum	[109]
Trichoderma orientalis	[35, 60]
Trichoderma reesei	[110, 111]
Trichoderma viride	[112]

concentrations, glycosidic linkages, and the architecture of glycosyl side chains, in addition to the two reactive hydroxyl groups at the xylose repetitions [114]. The second-most prevalent carbohydrate in lignocellulosic is called xylan, which is a hetero-polysaccharide made up of 1,4-β-xylose monomers with different substituents [115]. As seen in Fig. 14.6, a large number of hydrolytic proteins are necessary for the complete breakdown of xylan, with xylanase playing a key role. The enzyme xylanase breaks down xylan to create oligosaccharides, which 1,4-β-xylosidase subsequently breaks down to create xylose. To fully hydrolyse xylans, a number of other enzymes like ferulic and p-coumaric esterases, xylan esterases, α-4-O-methyl glucoronosidases, and α-1-arabinofuranosidases collaborate. Mannans and heteromannanas are additional polysaccharides that contribute to the hemicellulose of plant cell walls in addition to xylanase. D-Mannose, a six-carbon sugar, is the primary component of mannan, but because plant mannans have a complex and heterogeneous structure, a combination of endo-1,4-β-mannanases and exo acting mannosidases as well as additional enzymes like β-glucosidases, α-galactosidases, and acetyl mannan is required for the complete breakdown. Therefore, the hydrolysis of various hemicellulosic structures involves the following enzymes.

As seen in Fig. 14.6, there are three main kinds of xylanases that degrade xylan.

1. **Endo-β-1,4-xylanase (EC 3.2.1.8):** This enzyme hydrolyses glycosidic linkages to liberate both linear and branched oligosaccharides, randomly breaking the xylan chain.

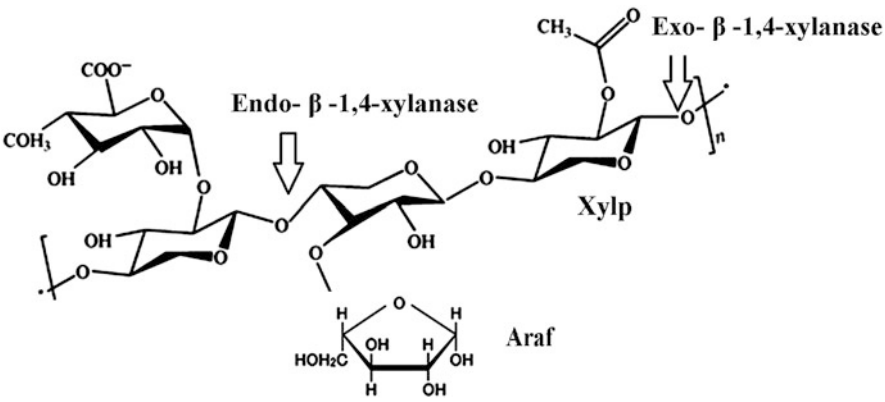

Fig. 14.6 Schematic representation of linkages in arabinoxylan and the enzymes hydrolysing the polysaccharide. Araf corresponds to arabinofuranose, and Xylp indicates xylopyranose in xylan [116]

2. **Exo-β-1,4-xylanase, also known as β-1,4-xylan xylohydrolase**: It is an enzyme that digests the non-reducing end of xylan polymers to eliminate monomeric xylose units.
3. **Xylobiase or β-1,4-xylosidase (EC 3.2.1.37)**: This enzyme hydrolyses xylobiose and elevated xylooligosaccharides, which are disaccharides with low specific affinities.

The following enzymes participate in the hydrolysis of mannans and galactomannans, as well as their modes of action are also depicted in Fig. 14.7:

1. **Endo-β-1,4-mannanase (EC 3.2.1.78):** It generates new chain ends by randomly rupturing the mannan's β-1,4-linkage internal links.
2. **Exo-β-mannosidase** (EC 3.2.1.25): It releases mannose sugar moieties by breaking β-1,4-linked mannosides from the non-reducing ends of mannan and mannooligosaccharides.
3. **β-Glucosidase (EC 3.2.1.21):** The oligosaccharide 1,4-β-D-glucopyranose present at the non-reducing ends of glucomannan and galactoglucomannan is hydrolysed by this enzyme.
4. **α-Galactosidase (EC 3.2.1.22):** The α-1,6-linked D-galactopyranosyl side chains of galactomannan and galactoglucomannan are hydrolysed by this debranching enzyme.
5. **Acetyl mannan esterase:** It is a de-branching enzyme that releases acetyl groups by breaking down galactoglucomannan.

Hemicellulases can be produced by a wide variety of bacteria. Some of the bacteria that make cellulases also co-produce these. The different fungi that generate hemicellulases are shown in Table 14.5.

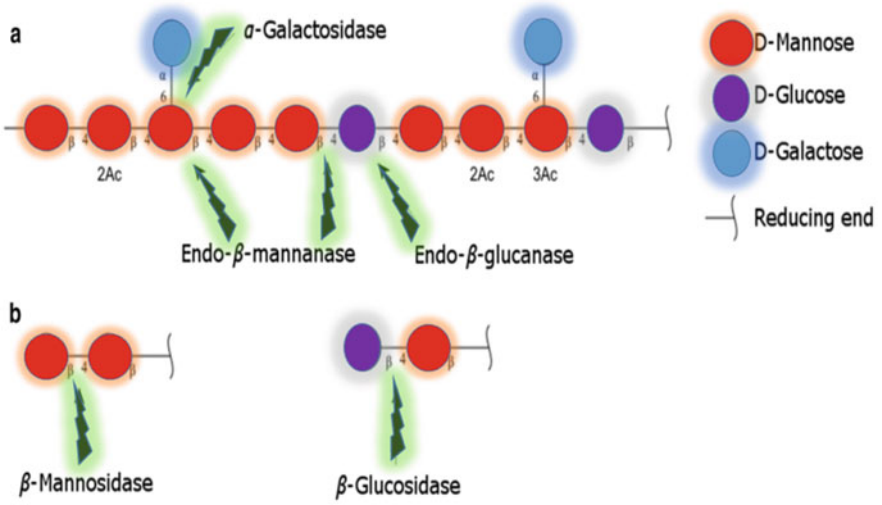

Fig. 14.7 Schematic representation of O-acetylated galactoglucomannan and enzymes involved in its degradation (**a**) and the oligosaccharides released (**b**) [117]

Table 14.5 List of various fungi producing hemicellulases

Fungal strain	Reference
Agaricus bisporus	[118]
Aspergillus aculeatus	[119]
Aspergillus awamori	[120]
Aspergillus fumigatus	[28, 121, 122]
Aspergillus niger	[31–33, 92, 123]
Aspergillus oryzae	[124]
Penicillium oxalicum	[102]
Aspergillus tamarii	[125]
Aspergillus terreus	[126, 127]
Ceriporiopsis subvermispora	[128]
Fusarium oxysporum	[94]
Fusarium solani	[96]
Penicillium chrysogenum	[129]
Penicillium echinulatum	[100]
Penicillium humicola	[130]
Penicillium purpurogenum	[131]
Penicillium roqueforti	[132, 133]
Pyrenophora phaeocomes	[77]
Trichoderma asperellum	[134–136]
Trichoderma harzianum	[108, 133, 137]
Trichoderma reesei	[138, 139]

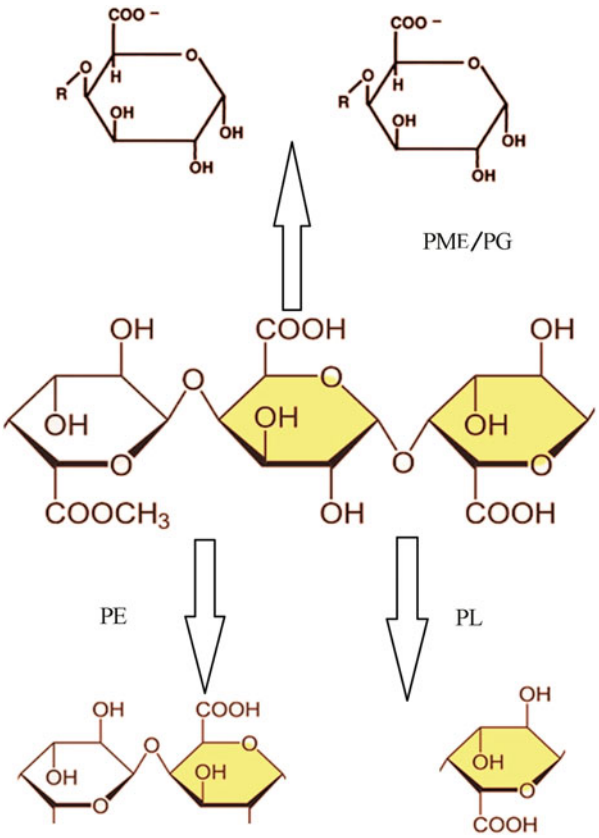

Fig. 14.8 Pectinases have the following modes of action: (**a**) R = H for PG and CH3 for PMG; (**b**) PE; and (**c**) R = H for PGL and CH for PL. The arrow points to the location where pectinase reacts with pectic compounds. PMG stands for polymethylgalacturonases; PG stands for polygalacturonases; PE stands for pectinesterase; and PL stands for pectin lyase [141]

14.3.3.1.3 Pectinases

Pectinases are the enzymes that hydrolyse pectic polysaccharides into monomers such as galacturonic acids. Pectinases are necessary to completely hydrolyse the pectic compounds since they are a part of the plant's cell wall. This lowers the medium viscosity and creates a favourable environment for the other enzymes to work on a variety of polysaccharides. The following are the primary enzymes involved in the hydrolysis of pectic compounds (Fig. 14.8) [140]:

1. **Proto-pectinases:** To release highly soluble polymerized pectin, they solubilize proto-pectin. They come in two different varieties: type A reacts with protopectin at the polygalacturonic acid chain region, while type B reacts with the polysaccharide chains linking the polygalacturonic acid chain with cell wall constituents.
2. **Pectin Methyl Esterases (PME) (EC 3.1.1.11):** Pectin methyl esterases deesterify the methyl group of pectin, releasing pectic acid and methanol in the process. Prior to pectatelyases and polygalacturonases, which need non-esterified substrates, it catalyses the de-esterification.

3. **Pectin Acetyl Esterases (PAE):** It catalyses the release of pectic acid and acetate from the acetyl esters that make up pectin.
4. **Polymethylgalacturonases (PMG):** It produces 6-methyl-D-galacturonate by breaking down α-1,4-glycosidic connections found in the pectin backbone. Both endo and exo mechanisms of action are possible. Exo-PMG catalyses the reaction at the non-reducing end of the substrate whereas endo-PMG randomly cleaves the substrate.
5. **Polygalacturonases (PG):** It creates D-galacturonate by cleaving the α-1,4-glycosidic bond in polygalacturonic acid. It can dissolve both endo and exobonds, much as PMG. Exo-PG (EC 3.2.1.67) catalyses the reaction at the non-reducing end, while Endo-PG (EC 3.2.1.15) cleaves the substrate at random.
6. **Pectate Lyases (PGL):** In order to liberate α-4,5-D-galacturonate from polygalacturonic acid, a trans-elimination process is used. While Exo-PGL (EC 4.2.2.9) cleaves the substrate at the non-reducing end, Endo-PGL (EC 4.2.2.2) acts on the substrate in a random manner.
7. **Pectin Lyases (PL):** By performing trans-elimination of glycosidic connections, it randomly cleaves the esterified pectin and creates unsaturated methyl-oligogalacturonates.

Pectinolytic enzymes are produced by a variety of plant-pathogenic fungi, which aid in their invasion of the host. Additionally, they help nature recycle carbon molecules by decomposing dead plant debris. Pectinases are made by a number of fungus, including *Aspergillus*, *Fusarium*, and *Penicillium* [142]. The different fungi that generate pectinases are shown in Table 14.6.

Table 14.6 List of various fungi-producing pectinases

Fungal strain	References
Aspergillus fumigatus	[28, 122]
Aspergillus niger	[31–33, 143, 144]
Aspergillus oryzae	[145, 146]
Aspergillus parvisclerotigenus	[147]
Aspergillus sojae	[148]
Aspergillus tamarii	[149]
Aspergillus tubingensis	[150]
Fusarium moniliforme	[151]
Fusarium oxysporum	[152]
Penicillium chrysogenum	[153]
Penicillium digitatum	[154–156]
Penicillium echinulatum	[100]
Penicillium expansum	[152]
Penicillium janczewskii	[157]
Penicillium janthinellum	[158]
Penicillium oxalicum	[159]
Trichoderma reesei	[160, 161]
Trichoderma viride	[162]

14.4 Third-Generation Biofuels

Microalgae are the source of third-generation biofuel, also known as algal fuels or microbiofuels, which is a practical alternative energy source that solves the significant drawbacks of first- and second-generation biofuels [163]. As a result, microalgae-based biofuels are the state-of-the-art biofuels that are now the most pressing issue in the market for renewable energy and are anticipated to replace fossil fuels. Due to their greater photosynthetic efficiency and quicker growth than any other plant, algae are increasingly recognized as an unconventional biofuel source. By employing wastewater as a culture medium and cultivating microalgae on bare land, microalgae can help regulate the level of water sources [164]. Algae may create biomass in three distinct ways throughout a fermentation process: autotrophic, heterotrophic, and mixotrophic [165]. From this biomass, enormous quantities of proteins, lipids, and carbohydrates are produced [166]. The idea of a biorefinery was developed as a result of the fact that these biological materials may be converted into profitable co-products and biofuel [165]. Compared to conventional crops, it can yield more biofuel per acre. The biomass from algae may also be used to make biodiesel and bioethanol. Among the numerous algae species are *Botryococcus braunii*, *Chaetoceros calcitrans*, *Chlorella* spp., *Isochrysis galbana*, *Nannochloropsis* sp., and *Schizochytrium limacinum*. Researchers looked for an alternate substrate for biofuel production in light of the drawbacks of both first- and second-generation biofuels, which led to the discovery of microalgae potential. Due to its excellent lipid synthesis efficiency, algal biomass, including micro- and macro-algae, has been extensively studied and evaluated as a substrate for third-generation biofuels. After just 5 to 6 days, microalgae may be removed and continue to proliferate [167]. Because microalgae may be grown in moist, marginal environments or wastewater, their cheaper growth cost is the main advantage of using them as a substrate. Because microalgae farming uses minimal land and generates large amounts of oil, oxygen, and hydrogen, it is ecologically friendly [168]. Temperature, CO2 concentration, illumination, pH, and the amount of nutrients in the growth medium all have an impact on how microalgae develop [169].

Autotrophic (uses inorganic carbon), heterotrophic (uses organic carbon), and mixotrophic (uses both inorganic and organic carbon) are the three primary categories of microalgal species [170]. In mixotrophic microalgae, autotrophic and heterotrophic microalgae coexist. As a result, biomolecule synthesis and the ability to adapt to growth in dark effluent are encouraged. Lipids are one of the most useful biomolecules in the production of biofuel and may be converted into it either through a thermochemical or biochemical process. Thermochemical procedures are frequently employed because of their higher conversion efficiency, less batch processing, and lower costs [170].

Spirogyra and *Chlorococcum* are two examples of green algae species that include polysaccharides in their cell walls. *Chlorococcum vulgaris* is the ideal choice for bioethanol since it has a conversion efficiency of 65% and contains around 37% starch by dry weight [171, 172]. Such polysaccharide-based feedstock needs intensive pretreatment and saccharification processing before fermentation

[173]. As discussed with first-generation biofuels, ethanol may be produced in a single step by consecutively performing saccharification and fermentation with an amylase enzyme producing strain.

14.5 Fourth-Generation Biofuels

Due to its cheap cultivation costs and high oil and fat production, third-generation biofuel has the potential to replace fossil fuels with renewable ones. Additionally, the drying of microalgae and the running expenses of lipid extraction need a sizable quantity of energy. Algal fuels or microbiofuels, which are fourth-generation biofuels made from bioengineered microalgae, are seen as sustainable and exciting alternatives to non-renewable fuels [174, 175]. These microorganisms have undergone bioengineering in order to enhance the amount of CO_2 they take up during photosynthesis, creating a carbon sink and boosting the production of bioethanol [176]. Many microalgae strains, including *Chlamydomonas reinhardtii*, *Phaeodactylum tricornutum*, and *Thalassiosira pseudonana*, have undergone physiological engineering to speed up their rate of development and adaption in constrained conditions [176]. Bioengineered microalgae offer environmental benefits such as CO_2 absorption and assimilation, medium for wastewater treatment, and GHG reduction [177]. The fourth generation of biofuel aims to have a smaller environmental effect than earlier generations, even if innovation is still in its early stages. Both an open and a closed system may be used to grow genetically modified microalgae [178]. Closed systems are more dependable than open systems because they protect the cultivation system from the outside environment and reduce pollutants [178]. The closed system, on the other hand, has a higher operational cost, which makes it less financially viable. Since the open system is prone to spillage, microalgae may escape or be excreted into the surroundings [179].

14.6 Commercial Enzymes and Advancement of Research for Production of Biofuels

Currently, high carbohydrate biomasses from sugar cane or cereal grains, or, more recently, cellulose and hemicellulose biomasses, are used to ferment sugar to create bioethanol. The demand for enzymes across the world is met by around 12 big suppliers and 400 smaller ones. The top three enzyme producers—Novozymes, Denmark's America's DuPont (after its acquisition of Denmark's Danisco in May 2011), and Switzerland's Roche—produce around 75% of all enzymes. The market is very competitive due to low profit margins and a high level of technological proficiency. Hydrolytic enzymes make up around 75% of all commercial enzymes. More than 70% of all enzyme sales are made up of carbohydrases, proteases, and lipases, which control the enzyme market [180]. In a wide range of industrial processes, including the creation of food and beverages, biofuels, household cleaners, and other goods, ethanol enzymes are extensively and widely employed.

Alcohol enzymes are in limited supply because of how many different industrial goods they may be used to. The combined market for alcohol and starch/sugar enzymes is anticipated to reach $2238.4 million in 2018 and increase at a 7.9% compound annual growth rate [180]. Amylases, proteases, lipases, cellulases, xylanases, and catalases are a few of the commercial enzymes that are employed in industries. Since starch is so widely available, α-amylases stood out as one of the most adaptable enzymes in the commercial enzyme market. They may be used to convert starch, make sugar syrup, or even create cyclodextrins for the pharmaceutical business. To increase starch gelatinization, decrease media viscosity, accelerate catalytic processes, and reduce the risks of bacterial contamination as their application spectrum expands, research is focused on developing novel α-amylases with greater thermophilic, thermotolerant, and pH-tolerant qualities. Table 14.7 provides a list of the widely manufactured amylase preparations.

A crucial enzyme in the creation of all first-generation biofuels is glucoamylase (GA). Multiple genome sequences being available have led to the identification of various new GA in recent years (Table 14.8). Finding better glucoamylases is crucial despite the fact that the relationship between sequence and functionality is still not totally understood. Novozymes recently cloned and characterized over 100 GAs with considerable variability (down to 40–50% identity) in an effort to improve glucoamylases. Even throughout the characterization process, more emphasis was given to "application-wise" character traits rather than conventional criteria like temperature and pH profiles, such as ethanol tolerance, activation at high substrate concentrations, and affinity for branched oligosaccharides. Numerous novel glucoamylases were also discovered, many of which possessed distinctive and crucial to industry properties, such as improved thermostability. Some of the commercially available glucoamylases are included in Table 14.7.

There are multiple time-consuming steps and various variables that must be meticulously controlled during the conversion of starch to sugars. Numerous issues in the starch industry are brought on by these variances. In recent years, several pullulanases, particularly those from thermophilic bacteria, have been discovered. The study of thermophile pullulanases is important for developing enhanced enzymes that may be used much more effectively in the industrial starch hydrolysis process as well as for understanding the factors governing enzyme stability. Pullulan was broken down by a maltogenic amylase created by Oh et al. [185] mostly into maltose and glucose with a small amount of panose. In summary, this is the first evidence of the presence of a specific de-branching enzyme that may produce a significant quantity of glucose from pullulan in addition to starch and maltooligosaccharides.

Enzymes called inulinases have the ability to attach to cellular membranes and release some of their contents into the extracellular space. They evolved from filamentous fungus and yeast. INU genes encode inulinases, another class of inducible enzymes [186]. Contrary to certain bacterial inulinases, which exhibit activity at higher pHs, inulinases derived from filamentous fungus have shown optimal pH activity spanning from 4.5 to 6.0. Most inulinases prefer temperatures between 30 and 55 °C; however higher temperatures have occasionally been seen. At very

Table 14.7 List of commercial preparations of amylases

Supplier	Common name	Trade name	Origin	Optimum pH	Optimum temperature °C	Characteristics
Genencor	α-Amylase	Purastar®	*B. licheniformis*	6–9	75–90	Thermostable
		Purastar® OxAm	GM Purastar	6–9	75–90	Thermostable, oxidatively thermostable
		GC 420	–	5.5–6.5	85–95	–
Novozymes		BAN®	*B. amyloliquefaciens*	6–6.5	70–90	–
		Termamyl®	*B. licheniformis*	6–9	70–90	Thermostable
		Duramyl®	GM Termamyl	6–9	65–85	Thermostable, oxidatively thermostable
		Termamyl® ultra	GM Termamyl	–	–	Reduced calcium dependency
		Stainzyme®	GM α-amylase	–	–	Altered substrate specificity
		Liquozyme SC DS	–	5.7–6.0	82–86	Thermostable
Novo Nordisk Biochem, N.C.	Glucoamylase	AMG 300 L	*Aspergillus niger*	–	–	–
Solvay enzymes, Indiana		Diazyme L-200	*Aspergillus niger*	–	–	–
Enzyme Biosystem limited, N.Y		G-Zyme	*Aspergillus niger*	–	–	–
Novozymes		Spirizyme Fuel	–	3.5–4.5	65–70	Thermostable
Novozymes, Bagsvaerd, Denmark	Pullulanase	Promozyme™ 400 L	*Bacillus acidopullulyticus*	–	–	–
Genencor	Glucoamylase and pullulanase	Distillase VHP	–	4.0–4.5	60	Thermostable
Novozymes, Bagsvaerd, Denmark	Inulinase	Fructozyme L™ Inulase	*Aspergillus niger*	–	–	–

Table 14.8 Sequenced genomes with new sources of glucoamylases [181]

Source	References	Characteristics
Endophytic fungus EF6	[182]	Higher activity on maltose than soluble starch
Monascus anka	[183]	–
Saccharomycopsis fibuligera R64	[184]	–
Talaromyces stipitatus	[126]	High expressibility in *Pichia pastoris*

high temperatures, they can be utilized to create inulin oligosaccharides from inulin polymer. Table 14.7 displays the commercial inulinase preparation.

Cellulases and hemicellulases are frequently used in the production of biofuels to break down pretreated lignocellulosic feedstocks such as maize stover, softwood pulp, and agricultural waste into fermentable sugars. In contrast, the starch-based ethanol industry has used biomass-decomposing enzymes including xylanases, cellobiohydrolases, and β-glucanases to increase production efficiency and energy consumption. A cellulase, hemicellulase, or their combination was previously added either before or during liquefaction to reduce the stickiness of the slurry [50, 187].

Recent technical advancements and methods have been developed to break down the maize kernel fibre matrix, increasing worldwide ethanol output. Cellulase cocktails, specialized equipment, and preprocessing techniques have been coupled to convert grain fibre into fermentable sugars for further ethanol production. Another new product on the market, "Spirizyme Achieve", provides improved process economics without necessitating a substantial financial outlay or process modifications. With increased cellulase activity, it is a saccharification enzyme that disintegrates the maize fibre network and releases bound starch that regular glucoamylases are unable to access. For ethanol producers, this has led to significant increases in ethanol yield [188].

Due to their wide range of uses, cellulases are already well-known. Cellulase is produced by a number of businesses for use, among other things, in papermaking, detergents, and textiles. The two leading producers of cellulase globally are "Genencor" and "Novozymes". Both companies have significantly reduced the cost of cellulases through cutting-edge research and are continuing to do so through the use of cutting-edge technology. Genencor has unveiled the cellulase complex Accelerase®1500, designed specifically for biomass processing facilities. It is viewed as more economical and efficient in the bioethanol industry than its predecessor, Accelerrase®1000. A *T. reesei* strain that has undergone genetic engineering is the source of Accelerase®1500 [189]. It is claimed that this enzyme formulation has more β-glucosidase potency than any other. According to claims, this enzyme formulation has a higher β-glucosidase potency than any other commercially available cellulase, permitting a conversion of almost 100% of cellobiose to glucose. When combined with other Accellerase® enzyme products, the Accellerase® XY auxiliary xylanase enzyme complex improves the transformation of both xylan (C5) and glucan (C6). Accellerase® XC is an auxiliary xylanase/cellulase enzyme complex with a broad profile of hemicellulase and cellulase activity that promotes

Table 14.9 Cellulases available in the market and their origin [189]

Enzyme samples	Company/supplier	Microbial sources
Accelerase®1500	Genencor	*T. reesei*
Bio-feed beta L, Energex L, Ultraflo L, Viscozyme L	Bio-feed beta L, Energex L, Ultraflo L, Viscozyme L	Bio-feed beta L, Energex L, Ultraflo L, Viscozyme L
Biocellulase	A Quest Intl.	*A. niger*
Biocellulase TRI	Quest Intl. (Sarasota, FL	*T. reesei/T. longibrachiatum*
Cellubrix (Celluclast), Novozymes 188	Novozymes, Denmark	*T. longibrachiatum* and *A. niger*
Cellulase AP30K	Amano Enzyme	*A. niger*
Cellulase TAP106	Amano Enzyme (Troy, VA)	*T. viride*
Cellulase TRL	Solvay Enzymes (Elkhart, IN	*T. reesei/T. longibrachiatum*
Cellulase 2000 L	Rhodia-Danisco (Vinay, France)	*T. longibrachiatum/T. reesei*
Cellulyve	50 L Lyven (Colombelles, France)	*T. longibrachiatum/T. reesei*
Econase CE	Alko-EDC (New York, NY	*T. reesei/T. longibrachiatum*
GC 440	Genencor-Danisco (Rochester, USA)	*T. longibrachiatum/T. reesei*
GC 880	Genencor	*T. longibrachiatum/T. reesei*
Multifect CL	Genencor Intl. (S. San Francisco, CA)	*T. reesei*
Rohament CL	Rohm-AB Enzymes (Rajamaki, Finland)	*T. longibrachiatum/T. reesei*
Ultra-low microbial (ULM)	Iogen (Ottawa, Canada)	*T. reesei/T. longibrachiatum*
Viscostar 150 L	Dyadic (Jupiter, USA)	*T. longibrachiatum/T. reesei*
Viscozyme wheat	Novozymes	–

xylan (C5) and glucan (C6) conversion when combined with other Accellerase® enzyme products. When combined with cellulase products, the β-glucosidase enzyme Accellerase® BG improves glucan (C6) transformation. Some of the commercially available cellulases are included in Table 14.9. The majority of marketed cellulases are from *T. reesei* and *Aspergillus niger*; however *T. reesei* appears to be missing enough β-glucosidase to produce appropriate and thorough hydrolysis [190]. Cellobiose accumulated as a result of inadequate β-glucosidase inhibition of exo- and endo-glucanases, which led to incomplete transformation [191]. Table 14.9 lists the cellulases now on the market along with where they came from.

Although the majority of the strains examined up to this point have been wild-type, efforts have been undertaken to lower the cost of the enzyme by locating overproducer mutants. Additionally, xylanase production in *Aspergillus* strains can be improved by parasexual recombination between overproducing strains [192]. Additionally, genetic modification has been successfully used and will soon play a bigger role in the excessive xylanase synthesis in many host species. However, as shown in Table 14.10, the majority of xylanase preparations continue to

Table 14.10 Examples of commercial preparations containing xylanases and mannanase [193–195]

Company	Product	Strain and mode of fermentation
Amano Pharmaceutical Co, Ltd. (Japan)	Amano 90	*Aspergillus niger* (SSF)
Gamma Chemie GmbH (Germany)	Gammafeed X, Gammazym X400OL	*Trichoderma longibrachiatum* (SmF); *Trichoderma reesei (SSF)*
Genencor	Optimash BG	–
Genencor International Europe Ltd. (Finland)	Multifect XL	*Trichoderma longibrachiatum* (SmF)
Hankyo Bioindustry Co. Ltd. (Japan)	Xylanase 250, Hemicellulase 100	*Trichoderma viride* (SSF) *Aspergillus niger* (SSF)
Quest International Ireland (Ireland)	Bioxylanase	*Trichoderma reesei* (SmF)
Röhm GmbH (Germany)	Rohalasa 7118, Vernon 191	*Aspergillus* sp. and *Trichoderma* sp. (SmF).
Shin Nihon Chemical (Japan)	Sumizyme X	*Trichoderma koningii* (SSF)
Solvay Enzymes GmbH & Co. (Germany)	Solvay Pentosanasa	*Trichoderma reesei* (SmF)
Stern-Enzym GmbH & Co. (Germany)	Sternzym HC 46; Sternzym HC 40	*Trichoderma reesei* (SmF) *Aspergillus niger* (SmF)/(SSF)

SmF submerged fermentation, *SSF* solid stated fermentation

come from spontaneously overproducing microorganisms. *Trichoderma* and *Aspergillus* species are typically used to make commercial xylanases [193]. Given the recent discovery of multiple prospective xylanase producers, this might alter in the future. In addition to having increased thermostability and acidic or alkaline stability, these enzymes also have higher catalytic activity. In order to affect the profitability of the xylanase production chain, significant progress has been achieved in identifying processing factors that lead to higher levels of xylanase output.

Recent research has focused on the cloning and modification of microbial mannanase genes from previously unknown organisms with the goals of increasing enzyme production, attempting to understand the primary structure of the protein, and using protein engineering to modify the properties of the enzyme to support industrial applications [196]. The majority of fungal-mannanases have been found in fungi like *P. pastoris* and *Aspergillus* sp. [197, 198]. One of the most crucial foundational elements needed for a future powered by renewable energy sources is enzymes. According to recent studies, there are several potential chances for more enzyme innovation in the realm of advanced biofuels.

14.7 Conclusion and Future Outlook

Major problems have emerged as a result of the transition to a smarter society, including i) fossil fuel depletion, which has led to rising prices; ii) global warming as a result of the indiscriminate use of these fuels; and iii) the unscientific disposal of urban and agricultural waste leftovers. These issues have caused the scientific community to focus on biofuels, which are made from a variety of biomass wastes, such as municipal and agricultural waste. Bioethanol and bio-CNG have the highest levels of acceptance among the various biofuels as substitutes for gasoline and CNG, respectively. The commercial manufacture of bioethanol, which is now the highest volume industrial fermentation product, generally uses sweet and starchy substrates. Commercial amylases, such as α-amylase and glucoamylase preparations, are utilized to target the starchy residues in the production of this first-generation bioethanol. However, because such starchy leftovers may be used as sustenance for humans, authorities are wary about their usage. Many nations have even imposed limitations on their usage, only permitting the use of ruined starchy grains and tubers that have turned rancid due to poor storage conditions. There is a need to look for next-generation amylases that can target even the non-gelatinized starch in order to minimize the processing cost. The synthesis of ethanol from grains requires one energy-intensive step of gelatinization to open up the crystalline structure of starch. As cellulose, hemicellulose, inulin, and pectin are among the polysaccharides found in starchy grains, it is necessary to utilize a mix of enzymes that can concurrently target both starchy and non-starchy carbohydrates in such cereals. Scientists are focusing on the use of agricultural, agro-industrial, and municipal solid waste as second-generation biofuel feedstocks as the biofuel industry develops because of the rise in demand for ethanol. Few businesses use these feedstocks for pretreatment and hydrolysis, which results in higher costs and severe chemical loading that eventually finds its way into our bodies and the environment. Due to their high cost and poor efficiency, enzyme use has not yet been commercialized. The hydrolysis of different carbohydrate residues in such feedstocks also necessitates the use of multiple enzymes. We need to lower the price of cellulases and hemicellulases or the industries must set up their own internal production of such enzymes if the second generation of bioethanol is to become a reality. Exploring microbial diversity or creating strains using metabolic engineering to increase productivity are the answers to all enzyme crises. The development of pH and temperature-compatible multiple enzyme consortia, either from a single or group of co-cultured microorganisms, is one of the key areas of scientific research in order to shorten the production timeline employing simultaneous hydrolysis and fermentation technologies. The excessive pressure on ethanol blending in gasoline and other biofuels around the world has turned the research attention to strain and process optimization for all the enzymes needed for effective conversion of agricultural and municipal waste. A new industrial revolution that evolved as a replacement for fossil fuels, renewable energy sources, was sparked by the development of biofuels. The development of effective and environment-friendly process technology for turning lignocellulosic residues into ethanol may be made possible by advancements in enzyme technology and

commercialization. This technology may prove to be a panacea for the major global problems of fossil fuel depletion and proper disposal of these priceless resources.

References

1. Van Der Maarel MJEC, Van Der Veen B et al (2002) Properties and applications of starch-converting enzymes of the α-amylase family. J Biotechnol 94:137–155
2. Tizhe DT, Jáafar JA (2021) Process parameters optimization for amylase production through rice bran bioconversion by *Aspergillus niger*. Afr J Biol Sci 3(3):41–47
3. Oh KW, Kim MJ et al (2005) Enzymatic characterization of a maltogenic amylase from *Lactobacillus gasseri* ATCC33323 expressed in *Escherichia coli*. FEMS Microbiol Lett 252: 175–181
4. Hossain M, ZabedAkter S et al (2020) Biogas from microalgae: technologies, challenges and opportunities. Renew Sust Energ Rev 117:109503
5. Khan A, Singh P, Srivastava A (2020) Microbial biofuels: an economic and eco-friendly approach. In: Kumar N (ed) Biotechnology for biofuels: a sustainable green energy solution. Springer, Singapore, pp 165–196
6. Kim JR, Karthikeyan KG (2021) Effects of severe pretreatment conditions and lignocellulose-derived furan byproducts on anaerobic digestion of dairy manure. Bioresour Technol 340: 125632
7. Sutaoney P, Choudhary R, Gupta AK (2020) Bioprospecting cellulolytic fungi associated with textile waste and invitro optimization of cellulase production by *Aspergillus flavus* NFCCI-4154. Development 13:64–84
8. Zhang Y, Yang J et al (2020) Low-cost Cellulase-Hemicellulase mixture secreted by *Trichoderma harzianum* EM0925 with complete Saccharification efficacy of lignocellulose. Int J Mol Sci 21(2):371
9. Kumar YS, Varakumar S, Reddy OV (2010) Production and optimization of polygalacturonase from mango (*Mangiferaindica* L.) peel using *Fusarium moniliforme* in solid state fermentation. World J Microbiol Biotechnol 26:1973–1980
10. Alalwan HA, Alminshid AH, Aljaafari HA (2019) Promising evolution of biofuel generations. Subject review Renew Energy Focus 28:127–139
11. Kopp M, Coleman D et al (2017) Energiepark Mainz: Technical and economic analysis of the worldwide largest Power-to-Gas plant with PEM electrolysis. Int J Hydrog Energy 42(19): 13311–13320
12. Scelsi E, Angelini A, Pastore C (2021) Deep eutectic solvents for the valorisation of lignocellulosic biomasses towards fine chemicals. Biomass 1(1):29–59
13. Gunaratne A, Corke H (2016) Starch, analysis of quality. Ref Module Food Sci 3:202–212
14. Saroj P, Manasa P, Narasimhulu K (2021) Assessment and evaluation of cellulase production using ragi (*Eleusine coracana*) husk as a substrate from thermo-acidophilic *Aspergillus fumigatus* JCM 10253. Bioprocess Biosyst Eng 44:113–126
15. Bertoldo C, Duffner F et al (1999) Pullulanase type I from *Fervidobacterium pennavorans* Ven5: cloning, sequencing, and expression of the gene and biochemical characterization of their recombinant enzyme. Appl Environ Microbiol 65:2084–2091
16. Kim CH, Nashiru O, Ko JH (1996) Purification and biochemical characterization of pullulanase type I from *Thermus caldophilus* GK-24. FEMS Microbiol Lett 138:147–152
17. Koch R, Canganella F et al (1997) Purification and properties of a thermostable pullulanase from a newly isolated thermophilic anaerobic bacterium, *Fervidobacterium pennavorans* Ven5. Appl Environ Microbiol 63:1088–1094
18. McKee LS, Peña MJ et al (2012) Introducing endo-xylanase activity into an exo-acting arabinofuranosidase that targets side chains. Proc Natl Acad Sci 109:6537–6542

19. Rogers J (2013) Going deep with enzyme technology-no part of the corn kernel goes unexplored. Ethanol Prod Mag:64–65
20. United Nations (2011) World population prospects: The 2010 Revision, Highlights and Advance Tables, Available at: http://esa.un.org/unpd/wpp/Documentation/pdf/WPP2010_Highlights.pdf
21. Duffner F, Bertoldo C et al (2000) A new thermoactive pullulanase from *Desulfurococcus mucosus*: cloning, sequencing, purification, and characterization of the recombinant enzyme after expression in *Bacillus subtilis*. J Bacteriol 182:6331–6338
22. Zinoviev S, Muller-Langer F et al (2010) Next-generation biofuels: survey of emerging technologies and sustainability issues. Chem Sus Chem 3:1106–1133
23. Bertoldo C, Antranikian G (2002) Starch-hydrolzing enzymes from thermophilic archaea and bacteria. Curr Opin Chem Biol 6:151–160
24. Karam EA, Wahab WAA et al (2017) Production, immobilization and thermodynamic studies of free and immobilized *Aspergillus awamori* amylase. Int JBiol Macromol 102:694–703
25. Adejuwon AO, Tsygankova VA, Alonge O (2021) Effect of cultivation conditions on the activity of α-amylase from a tropical strain *Aspergillus flavus* link. J Microbiol Biotechnol Food Sci 7(6):571–575
26. Mondal S, Soren JP et al (2020) Contemporaneous synthesis of multiple carbohydrate debranching enzymes from newly isolated *Aspergillus fumigatus* SKF-2 under solid state fermentation: a unique enzyme mixture for proficient saccharification of plant bioresources. Ind Crop Prod 150:112409
27. Bano S, Iqbal S et al (2021) Purification and characterization of [beta]-galactosidase from *Aspergillus fumigatus* PCSIR-2013. Pak J Pharm Sci 34(4):1333–1341
28. Mohan R, Subramanian R et al (2019) Enhancement of α-amylase production in pelleted *Aspergillus tamarii* through optimization for desizing of cotton fabric. J Environ Biol 40: 1084–1093
29. Bansal N, Tewari R et al (2011) A novel strain of *Aspergillus niger* producing a cocktail of hydrolytic depolymerising enzymes for the production of second generation biofuels. BioResources 6:552–569
30. Bellaouchi R, Abouloifa H et al (2021) Characterization and optimization of extracellular enzymes production by *Aspergillus niger* strains isolated from date by-products. J Genet EngBiotechnol 19(1):1–8
31. Janveja C, Rana SS, Soni SK (2013a) Kitchen waste residues as potential renewable biomass resources for the production of multiple fungal carbohydrases and second generation bioethanol. J Technol Innov Renew Energy 2:186–200
32. Janveja C, Rana SS, Soni SK (2013b) Environmentally acceptable management of kitchen waste residues by using them as substrates for the co-production of a cocktail of fungal carbohydrases. Int J Chem Environ Eng Sys 4:20–29
33. Kaur A, Rishi V et al (2020b) A novel multi-enzyme preparation produced from *Aspergillus niger* using biodegradable waste: a possible option to combat heterogeneous biofilms. AMB Express 10:1–16
34. Thurston CF (1994) The structure and function of fungal laccases. Microbiology 140:19–26
35. Xu L, Sun K et al (2020) Laccase production by *Trametes versicolor* in solid-state fermentation using tea residues as substrate and its application in dye decolorization. J Environ Manag 270:110904
36. Balakrishnan M, Jeevarathinam G et al (2021) Optimization and scale-up of α-amylase production by *Aspergillus oryzae* using solid-state fermentation of edible oil cakes. BMC Biotechnol 21(1):1–1
37. Braia M, Cabezudo I et al (2021) An optimization approach to the bioconversion of flour mill waste to α-amylase enzyme by *Aspergillus oryzae*. Process Biochem 111:102–108
38. Hamdan NT (2021) Optimization of Paddy straw saccharification using α-amylase from *Aspergillus tamarii*. Nat Volatiles Essent Oils 28:8070–8076

39. Metsoviti MN, Papapolymerou G et al (2019) Comparison of growth rate and nutrient content of five microalgae species cultivated in greenhouses. Plan Theory 8(8):279
40. Niehaus F, Peters A et al (2000) Cloning, expression and biochemical characterization of aunique thermostable pullulan-hydrolysing enzyme from the hyperthermophilic archaeon *Thermococcus aggregans*. FEMS Microbiol Lett. 190:223–229
41. Biswas P, Bharti AK et al (2019) Bio-deinking of mixed office waste paper by *Penicillium citrinum* NCIM-1398 and its comparative study with conventional chemical deinking. Bioresources 14:6544–6557
42. Ahmed NN, Abd El Rahman HM (2021) Optimizing the production of pectinase of orange peel waste by *Penicillium chrysogenum* mf318506 using response surface methodology in submerged fermentation. J Microbiol Biotechnol Food Sci 7:e3931
43. Mostafa E, Abo Elsoud M et al (2021) Optimization of aeration and agitation for α-amylase production by *Aspergillus flavus* from water hyacinth. Int J Innov Sustain Dev 2(1):25–35
44. Gu LS, Tan MZ et al (2020) ARTP/EMS-combined multiple mutagenesis efficiently improved production of raw starch-degrading enzymes in *Penicillium oxalicum* and characterization of the enzyme-hyperproducing mutant. Biotechnol Biofuels 13(1):1–2
45. Marques NP, de Cassia PJ et al (2018a) Cellulases and xylanases production by endophytic fungi by solid state fermentation using lignocellulosic substrates and enzymatic saccharification of pretreated sugarcane bagasse. Ind Crop Prod 122:66–75
46. Abdulaal WH (2018) Purification and characterization of α-amylase from *Trichoderma pseudokoningii*. BMC Biochem 19(1):1–6
47. Chi Z, Zhang T et al (2009) Inulinase-expressing microorganisms and applications of inulinases. Appl Microbiol Biotechnol 82:211–220
48. Chi ZM, Zhang T et al (2011) Biotechnological potential of inulin for bioprocesses. Bioresour Technol 102:4295–4303
49. Kango N, Jain SC (2011) Production and properties of microbial inulinases: recent advances. Food Biotechnol 25:165–212
50. Zehra M, Syed MN, Sohail M (2020) Banana peels: a promising substrate for the coproduction of pectinase and xylanase from *Aspergillus fumigatus* MS16. Pol J Microbiol 69:19–26
51. Prasoulas G, Gentikis A et al (2020) Bioethanol production from food waste applying the multienzyme system produced on-site by *Fusarium oxysporum* F3 and mixed microbial c ultures. Fermentation 6:39
52. Sunkar B, Kannoju B, Bhukya B (2020) Optimized production of xylanase by *Penicillium purpurogenum* and ultrasound impact on enzyme kinetics for the production of monomeric sugars from pretreated corn cobs. Front Microbiol 11:772
53. Luo Y, Li Z et al (2019) The production of furfural directly from hemicellulose in lignocellulosic biomass: a review. Catal Today 319:14–24
54. Rudiger A, Jorgensen PL, Antranikian G (1995) Isolation and characterization of a heat- stable pullulanase from the hyperthermophilic archaeon *Pyrococus woesei* after cloning and expression of its gene in *Escherichia coli*. Appl Environ Microbiol 61:567–575
55. Sathyanarayana NG, Panda T (2003) Purification and biochemical properties of microbial pectinases: a review. Process Biochem 38:987–996
56. Curran LMLK, Sale KL, Simmons BA (2021) Review of advances in the development of laccases for the valorization of lignin to enable the production of lignocellulosic biofuels and bioproducts. Biotechnol Adv 2021:107809
57. Singhania RR, Sukumaran RK et al (2010) Advancements and comparative profiles in production technologies using solid and submerged state fermentation systems for microbial cellulases. Enzym Microb Technol 46:541–549
58. Yahya S, Muhammad F et al (2021) Amylase production and growth pattern of two indigenously isolated *aspergilli* under submerged fermentation: influence of physico-chemical parameters. Pak J Bot 53(3):1147–1155
59. Gianfreda L, Xu F, Bollag JM (1999) Laccases: a useful group of oxidoreductive enzymes. Bioremdiat J J.3:1–25

60. Han J, Xue Y et al (2020) Effect of VIB gene on Cellulase production of *Trichoderma orientalis* EU7-22. Appl Biochem 1:12
61. Ricca E, Calabro V et al (2009) Optimization of inulin hydrolysis by inulinase accounting for enzyme time and temperature dependent deactivation. Biochem Eng J 48:81–86
62. Baldrian P, Snajdr J (2006) Production of ligninolytic enzymes by litter- decomposing fungi and their ability to decolorize synthetic dyes. Enzym Microb Technol 39:1023–1029
63. Claus H (2004) Laccases: structure, reactions, distribution. Micron 35:93–96
64. Thakur S, Gupte A (2015) Optimization and hyper production of laccase from novel agaricomycete *Pseudolagarobasidium acaciicola* AGST3 and its application in in vitro decolorization of dyes. Ann Microbiol 1:185–196
65. Asgher M, Wahab A et al (2016) Lignocellulose degradation and production of lignin modifying enzymes by *Schizophyllum commune* IBL-06 in solid-state fermentation. Biocatal Agric Biotechnol 6:195–201
66. Das A, Bhattacharya S et al (2016) Production, characterization and Congo red dye decolourizing efficiency of a laccase from *Pleurotus ostreatus* MTCC 142 cultivated on co-substrates of paddy straw and corn husk. J Gene Eng Biotechnol 14:281–288
67. Malhotra M, Suman SK (2021) Laccase-mediated delignification and detoxification of lignocellulosic biomass: removing obstacles in energy generation. Environ Sci Pollut Res 28:1–16
68. Bule MV, Chaudhary I et al (2016) Effects of extracellular proteome on wheat straw pretreatment during solid-state fermentation of *Phlebiaradiata* ATCC 64658. Int Biodeterior Biodegrad 109:36–44
69. Jin X, Ning Y (2013) Laccase production optimization by response surface methodology with *Aspergillus fumigatus* AF1 in unique inexpensive medium and decolorization of different dyes with the crude enzyme or fungal pellets. J Hazard Mat 262:870–877
70. Widjaja A, Chien CC, Ju YH (2009) Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. J Taiwan Inst Chem Eng 40:13–20
71. Sharma S, Bajaj BK (2017) Xylanase production from a new strain of *aspergillus terreus* S9 and its application for saccharification of rice straw using combinatorial approach. Environ Prog Sustain Energy 37:1210–1219
72. Peralta AG, Venkatachalam S et al (2017) Xylan epitope profiling: an enhanced approach to study organ development-dependent changes in xylan structure, biosynthesis, and deposition in plant cell walls. Biotechnol Biofuels 10:1–13
73. Karp SG, Faraco V et al (2015) Statistical optimization of laccase production and delignification of sugarcane bagasse by *Pleurotus ostreatus* in solid state fermentation. Biomed Res Int 2015:1
74. Junior JA, Vieira YA et al (2020) Sequential degradation of raw vinasse by a laccase enzyme producing fungus *Pleurotussajor caju* and its ATPS purification. Biotechnol Rep 25:e00411
75. Tarutina MG, Lazareva MN et al (2019) Expression of *Aspergillus aculeatus* β-mannanase in *Pichia pastoris* yeast and analysis of industrially important enzyme properties. Appl Biochem Microbiol 55:877–881
76. Hafid HS, Baharuddin AS et al (2021) Enhanced laccase production for oil palm biomass delignification using biological pretreatment and its estimation at biorefinary scale. Biomass Bioenerg 144:105904
77. Ramírez-Lagunes H, Aguilar-Uscanga MG et al (2021) Optimization of xylanase production from *Aspergillus tamarii* SCBH2 using response surface methodology. Biomass Convers Bioref 15:1–1
78. Li Q, Ray CS et al (2020b) *Aspergillus niger* production of pectinase and α-galactosidase for enzymatic soy processing. Enzym Microb Technol 134:109476
79. Xiang J, Wang X, Sang T (2021) Cellulase production from *Trichoderma reesei* RUT C30 induced by continuous feeding of steam-exploded *Miscanthus lutarioriparius*. Ind Crop Prod 160:113129
80. Ghorbani F, Karimi M et al (2015) Enhancement of fungal delignification of rice straw by *Trichoderma viride* sp. to improve its saccharification. Biochem Eng J 101:77–84

81. Kudanga T, Nyanhongo GS et al (2011) Potential applications of laccase-mediated coupling and grafting reactions: a review. Enzym Microb Technol 48:195–208
82. Bhat MK (2000) Cellulases and related enzymes in biotechnology. Biotechnol Adv 18:355–383
83. Baig KS (2020) Interaction of enzymes with lignocellulosic materials: causes, mechanism and influencing factors. Bioresour Bioprocess 7(1):1–19
84. Xiaoyu F, Huiying C et al (2013) Enhancement of laccase activity by marine-derived deuteromycete *Pestalotiopsis* sp. J63 with agricultural residues and inducers. Chinese J Chem Eng 21:1182–1189
85. Bansal N, Tewari R et al (2012) Production of cellulases from *Aspergillus niger* NS-2 in solid state fermentation on agricultural and kitchen waste residues. Waste Manag 32:1341–1346
86. Bansal N, Janveja C et al (2014) Highly thermostable and pH-stable cellulases from *Aspergillus niger* NS-2: properties and application for cellulose hydrolysis. App Biochem Biotechnol 172:141–156
87. Kazeem MO, Ajijolakewu KA et al (2021) Tamarind extract pretreatment: valorization of sugarcane bagasse for cellulase production by *aspergillus flavus*. Ife J Sci Technol 23(2): 25–34
88. Siamphan C, Arnthong J et al (2021) Production of D-galacturonic acid from pomelo peel using the crude enzyme from recombinant *Trichoderma reesei* expressing a heterologous exopolygalacturonase gene. J Clean Prod 129958
89. Stark A (2011) Ionic liquids in the biorefinery: a critical assessment of their potential. Energy Environ Sci 4(1):19–32
90. Dong M, Wang S et al (2019) Cellulase production by *Aspergillus fumigatus* MS13. 1 mutant generated by heavy ion mutagenesis and its efficient saccharification of pretreated sweet sorghum straw. Process Biochem 84:22–29
91. Saini A, Aggarwal NK (2020) Valorization of *Parthenium hysterophorus* biomass by its utilization in endoglucanase production by *Penicillium citrinum* NAF5. Waste Biomass Valor 11:2591–2601
92. Kaur J, Chugh P et al (2020a) A low-cost approach for the generation of enhanced sugars and ethanol from rice straw using in-house produced cellulase-hemicellulase consortium from *A. niger* P-19. Bioresour Technol Rep 11:100469
93. Shi L, Yu H et al (2014) Biochemical and molecular characterization of a novel laccase from selective lignin-degrading white-rot fungus *Echinodontium taxodii* 2538. Process Biochem 49: 1097–1106
94. Pourkhanali K, Khayati G et al (2021) Isolation, identification and optimization of enhanced production of laccase from *Galactomyces geotrichum* under solid-state fermentation. Prep Biochem Biotechnol 51(7):659–668
95. Hussein MA, Gherbawy Y, El-Dawy EG (2020) Characterization, pathogenicity and enzymatic profile of *Fusarium solani* associated with potato tubers in upper Egypt. Arch Phytopathol Plant Protect 53(11–12):495–508
96. Marques SF, Minafra CS et al (2018b) Production and characterization of a *Trichoderma harzianum m*ultienzyme complex and its application in broiler chicks' diets. Curr Biotechnol 7:26–33
97. Abdel-Sater M, Hussein N et al (2019) Immobilization of Cellulases produced by *Penicillium brevicompactum* AUMC 10987, using cross-linkage, chitosan-coating, and encapsulation. Int J Environ Sci 18(1):139–149
98. Saha BC, Qureshi N et al (2016) Biological pretreatment of corn Stover with white-rot fungus for improved enzymatic hydrolysis. Int Biodeterior Biodegrad 109:29–35
99. Natalia D, Vidilaseris K et al (2011) Biochemical characterization of a glucoamylase from *Saccharomycopsis fibuligera* R64. Biologia 66:27–32
100. Schenk PM, Thomas-Hall SR et al (2008) Second generation biofuels: high-efficiency microalgae for biodiesel production. Bioenergy Res 1:20–43

101. Nouadri T, Meraihi Z et al (2010) Purification and characterization of the α-amylase isolated from *Penicillium camemberti* PL21. Afr J Biochem Res 4:155–162
102. Lam MK, Lee KT (2012) Microalgae biofuels: a critical review of issues, problems and the way forward. Biotechnol Adv 30:630–690
103. Li CX, Zhao S et al (2020a) Weighted gene co-expression network analysis identifies critical genes for the production of Cellulase and xylanase in *Penicillium oxalicum*. Front Microbiol 11:520
104. Pagnonceli J, Rasbold LM et al (2019) Biotechnological potential of an exo-polygalacturonase of the new strain *Penicillium janthinellum* VI2R3M: biochemical characterization and clarification of fruit juices. J Appl Microbiol 127:1706–1715
105. Shah SH, Raja IA et al (2018) Potential of microalgal biodiesel production and its sustainability perspectives in Pakistan. Renew Sust Energ Rev 81:76–92
106. Gelain L, Kingma E et al (2021) Continuous production of enzymes under carbon-limited conditions by *Trichoderma harzianum* P49P11. Fungal Biol 125(3):177–183
107. Petrus J, Zyl V et al (2009) Production of the *Aspergillus aculeatus* endo-1,4-β-mannanase*in A. niger*. J Ind Microbiol Biotechnol 36:611–617
108. Von Freiesleben P, Spodsberg N et al (2018) Boosting of enzymatic softwood saccharification by fungal GH5 and GH26 endomannanases. Biotechnol Biofuels 11:194
109. Osman AI, Hefny M et al (2020) Recent advances in carbon capture storage and utilisation technologies: a review. Environ Chem Lett:1–53
110. Venditti T, D'hallewin G et al (2018) Effect of NaHCO3 treatments on the activity of cell-wall-degrading enzymes produced by *Penicillium digitatum* during the pathogenesis process on grapefruit. J Sci Food Agric 98:4928–4936
111. Xie G, Bruce DC et al (2007) Genome sequence of the cellulolytic gliding bacterium *Cytophaga hutchinsonii*. Appl Environ Microbiol 73:3536–3546
112. Ardhi MW, Muktiani E et al (2021) The effect of incubation time on various type of local agricultural waste in Madiun, Indonesia to produce Cellulases using *Trichoderma viride*. In: In10th international seminar and 12th congress of Indonesian society for microbiology. Atlantis Press, pp 164–174
113. Loera O, Cordova J (2003) Improvement of xylanase production by a parasexual cross between *Aspergillus niger* strains. Braz Arch Biol Technol 46:177–181
114. Yaropolov AI, Skorobogatko OV et al (1994) Laccase: properties, catalytic mechanism, and applicability. Appl Biochem Biotech 49:257–280
115. Papzan Z, Kowsari M et al (2021) Strain improvement of *Trichoderma spp*. through two-step protoplast fusion for cellulase production enhancement. Can J Microbiol 67(5):406–414
116. Matrawy AA, Khalil AI et al (2021) Use of wheat straw for value-added product xylanase by *Penicillium chrysogenum* strain A3 DSM105774. J Fungus 7(9):696
117. Vilanova L, López-Pérez M et al (2018) Differential contribution of the two major polygalacturonases from *Penicillium digitatum* to virulence towards citrus fruit. Int J Food Microbiol 282:16–23
118. Kabel MA, Jurak E et al (2017) Occurrence and function of enzymes for lignocellulose degradation in commercial *Agaricus bisporus* cultivation. Appl Microbiol Biotechnol 101: 4363–4369
119. Tangngamsakul P, Karnchanatat A et al (2011) An extracellular glucoamylase produced by endophytic fungusEF6.Appl. Biochem Microbiol 47:412–418
120. de Sousa Paredes R, de Barros RD et al (2015) Production of xylanase, α-L-arabino furanosidase, β-xylosidase, and β-glucosidase by *Aspergillus awamori* using the liquid stream from hot-compressed water treatment of sugarcane bagasse. Biomass Convers. Bioref. 5:299–307
121. Bernardi AV, Gerolamo LE, Uyemura SA et al (2021) A thermophilic, pH-tolerant, and highly active GH10 xylanase from *Aspergillus fumigatus* boosted pre-treated sugarcane bagasse saccharification by cellulases. Ind Crop Prod 170:113697

122. Yoshizaki Y, Susuki T et al (2010) Characterization of glucoamylase and α-amylase from *Monascus anka*: enhanced production of α-amylase in red koji. J Biosci Bioeng 6:670–674
123. Azzouz Z, Bettache A et al (2021) Optimization of β-1, 4-Endoxylanase production by an *Aspergillus niger* strain growing on wheat straw and application in Xylooligosaccharides production. Molecules 26(9):2527
124. Bhardwaj N, Verma VK et al (2020) Cloning, expression, and characterization of a thermo-alkali-stable xylanase from *Aspergillus oryzae* LC1 in Escherichia coli BL21 (DE3). Prot Exp Purif 168:105551
125. Rahim AHA, Khoo KS et al (2019) Ether-functionalized ionic liquids as solvent for *Gigantochloas cortechini* dissolution. In: AIP conference proceedings, vol 2157(1). AIP Publishing LLC, p 020025
126. Huang SP, Wang CL et al (2007) Construction of a double functional recombinant strain of *Pichia pastoris* coexpressing phytase and mannanase and the enzymatic analysis. Acta Microbiol Sin 47:280–284
127. Sharma N, Soni R et al (2016) Statistical optimization of Thermo-alkaline pretreatment of corn Stover for efficient enzymatic hydrolysis. J Mech Eng Sci Technol 3:4635–4640
128. Chmelová D, Ondrejovič M (2020) Determination of enzymes produced by *Ceriporiopsis subvermispora* during pretreatment of different biomass sources. J Microbiol Biotechnol Food Sci 9:1168–1178
129. Martínez-Pacheco MM, Flores-García A et al (2020) Optimization of production of xylanases with low cellulases in *Fusarium solani* by means of a solid state fermentation using statistical experimental design. Rev Argent Microbiol 52(4):328–338
130. Ismail SA, Khattab OKH et al (2019) A thermodynamic study of partially-purified *Penicillium humicola* β-mannanase produced by statistical optimization. Jordan J Biol Sci 12:209–217
131. Solomon EI, Sundaram UM, Machonkin TE (1996) Multicopper oxidases and oxygenases. Chem Rev 96:2563–2605
132. de Almeida Antunes Ferraz JL, Oliveira Souza L et al (2020) Optimization of the solid-state fermentation conditions and characterization of xylanase produced by *Penicillium roqueforti* ATCC 10110 using yellow mombin residue (*Spondias mombin* L.). Chem Eng Commun 207: 31–42
133. Marella TK, Tiwari A (2020) Marine diatom *Thalassiosira weissflogii* based biorefinery for co-production of eicosapentaenoic acid and fucoxanthin. Bioresour Technol 307:123245
134. Akinyele HA, Gabriel-Ajobiewe RA et al (2019) Xylanase-production potential of *Trichoderma asperellum* NG-T161 and NG-T163 isolated from banana farm soils in South Western Nigeria. Curr Res Environ Appl 9(1):301–312
135. Ezeilo UR, Lee CT et al (2019a) Raw oil palm frond leaves as cost-effective substrate for cellulase and xylanase productions by *Trichoderma asperellum* UC1 under solid-state fermentation. J Environ Manag 243:206–217
136. Ezeilo UR, Wahab RA, Mahat NA (2019b) Optimization studies on cellulase and xylanase production by *Rhizopus oryzae* UC2 using raw oil palm frond leaves as substrate under solid state fermentation. Renew Energy 156:1301–1312
137. Zeni J, Cence K et al (2011) Screening of pectinase-producing microorganisms with polygalacturonase activity. Appl Biochem Biotechnol 163:383–392
138. Cekmecelioglu D, Demirci A (2020) Production of cellulase and xylanase enzymes using distillers dried grains with solubles (DDGS) by *Trichoderma reesei* at shake-flask scale and the validation in the benchtop scale bioreactor. Waste Biomass Valor. 11:6575–6584
139. Xue Y, Han J et al (2020) Promoting cellulase and hemicellulase production from *Trichoderm aorientalis* EU7-22 by overexpression of transcription factors Xyr1 and Ace3. Bioresour Technol 296:122355
140. Pan Y, Gao L et al (2020) The role of cross-pathway control regulator CpcA in the growth and extracellular enzyme production of *Penicillium oxalicum*. Curr Microbiol 77:49–54

141. Satapathy S, Soren JP et al (2021) Industrially relevant pectinase production from *Aspergillus parvisclerotigenus* KX928754 using apple pomace as the promising substrate. J Taibah Univ Sci 15(1):347–356
142. Yousuf A, Pirozzi D, Sannino F (2020) Fundamentals of lignocellulosic biomass. In: Lignocellulosic biomass to liquid biofuels. Academic Press, pp 1–15
143. Begum G, Munjam S (2021) Carbon and nitrogen sources effect on pectinase synthesis by *Aspergillus niger* under submerged fermentation. Biosci Biotechnol Res Asia 18(1):185–195
144. Li H, Dou M et al (2021) Optimization of Cellulase production by a novel endophytic fungus *Penicillium oxalicum* R4 isolated from *Taxus cuspidata*. Sustainability 13(11):6006
145. Adedeji OE, Ezekiel OO (2019) Pretreatment of selected peels for polygalacturonase production by *Aspergillus awamori* CICC 2040: purification and application in mango juice extraction. Bioresour Technol Rep 7:100306
146. Ding C, Wang X, Li M (2019) Evaluation of six white-rot fungal pretreatments on corn Stover for the production of cellulolytic and ligninolytic enzymes, reducing sugars, and ethanol. Appl Microbiol 103:5641–5652
147. Sanyang ML, Ilyas RA et al (2018) Sugar palm starch-based composites for packaging applications. In: Bionano composites for packaging applications, pp 125–147
148. Fratebianchi D, Cavello IA, Cavalitto SF (2017) Purification and biochemical and kinetic properties of an endo-polygalacturonase from the industrial fungus *Aspergillus sojae*. J Mol Microbiol Biotechnol 27:102–109
149. Amande T, Adebayo-Tayo B et al (2021) Production and partial characterization of pectinases from mango peels by *Aspergillus tamarii*. J Microbiol Biotechnol Food Sci 3(1):59–62
150. Huang D, Song Y et al (2019) A new strain of *Aspergillus tubingensis* for high-activity pectinase production. Braz J Microbiol 50:53–65
151. Kumar P, Barrett DM et al (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res 48:3713–3729
152. Sukumaran RK, Singhania RR, Pandey A (2005) Microbial cellulases-production, applications and challenges. J Sci Ind Res 64:832–844
153. El Garhy GM, Azzaz HH et al (2020) Fungal pectinase production optimization and its application in Buffaloe's diets degradation. Int J Zoo Animal Biol 3:1–12
154. Li S, Yang X et al (2012) Technology prospecting on enzymes: application, marketing and engineering. Comput Struct Biotechnol J.2:e201209017
155. Vakulchuk R, Overland I, Scholten D (2020) Renewable energy and geopolitics: a review. Renew Sust Energ Rev 122:109547
156. Verma N, Kumar V (2020) Impact of process parameters and plant polysaccharide hydrolysates in cellulase production by *Trichoderma reesei* and *Neurospora crassa* under wheat bran based solid state fermentation. Biotechnol, Rep, p e00416
157. Amin F, Mohsin A et al (2020) Production, thermodynamic characterization, and fruit juice quality improvement characteristics of an Exo-polygalacturonase from *Penicillium janczewskii*. BBA-Proteins Proteom 1868(5):140379
158. Pachauri P, More S et al (2020) Purification and characterization of cellulase from a novel isolate of *Trichoderma longibrachiatum*. Biofuels 11:85–91
159. Cheng Z, Chen D et al (2017) Identification of an acidic endo-polygalacturonase from *Penicillium oxalicum* CZ1028 and its broad use in major tropical and subtropical fruit juices production. J Biosci Bioeng 123:665–672
160. Beier S, Hinterdobler W et al (2020) CLR1 and CLR2 are light dependent regulators of xylanase and pectinase genes in *Trichoderma reesei*. Fungal Genet Biol 136:103315
161. Siddiqa A, Noreen S et al (2018) Statistical optimization of pectin lyase from *Penicillium digitatum* in solid state fermentation. Int J Appl Biol Forensics 2:157–170
162. El-Shishtawy RM, Mohamed SA et al (2015) Saccharification and hydrolytic enzyme production of alkali pre-treated wheat bran by *Trichoderma virens* under solid state fermentation. BMC Biotechnol 15(1):1–3
163. Chisti Y (2007) Biodiesel from microalgae. Biotechnol Adv 25:294–306

164. Kim KH, Choi IS et al (2014) Bioethanol production from the nutrient stress-induced microalga *Chlorella vulgaris* by enzymatic hydrolysis and immobilized yeast fermentation. Bioresour Technol 153:47–54
165. Brennan L, Owende P (2010) Biofuels from microalgae: a review of technologies for production, processing, and extractions of biofuels and co-products. Renew Sust Energ Rev 14:557–577
166. Wang H, Zhai L, Geng A (2020) Enhanced cellulase and reducing sugar production by a new mutant strain *Trichoderma harzianum* EUA20. J Biosci Bioeng 129:242–249
167. Mann J, Markham JL et al (2015) Use of olive mill wastewater as a suitable substrate for the production of laccase by *Cerrena consors*. Int Biodeterior Biodegrad 99:138–145
168. Shah SH, Raja IA et al (2016) Improvement in lipids extraction processes for biodiesel production from wet microalgal pellets grown on diammonium phosphate and sodium bicarbonate combinations. Bioresour Technol 214:199–209
169. Messaoud EB, BenAmmar Y et al (2002) Thermostable pullulanase type I from new isolated *Bacillus thermoleovorans* US105: cloning, sequencing and expression of the gene in *E.coli*. Enzym Microb Technol 31:827–832
170. Schneider WD, Gonçalves TA et al (2018) Comparison of the production of enzymes to cell wall hydrolysis using different carbon sources by *Penicilliume chinulatum* strains and its hydrolysis potential for lignocelullosic biomass. Process Biochem 66:162–170
171. Eshaq FS, Ali MN, Mohd MK (2010) Spirogyra biomass a renewable source for biofuel (bioethanol) production. Int J Eng Sci Technol 2:7045–7054
172. Kumari D, Singh R (2018) Pretreatment of lignocellulosic wastes for biofuel production: a critical review. Renew Sust Energ Rev 90:877–891
173. Harun R, Danquah MK, Forde-Gareth M (2010) Microalgal biomass as a fermentation feedstock for bioethanol production. J Chem Technol Biotechnol 85:199–203
174. Ganesh I (2014) Conversion of carbon dioxide into methanol-a potential liquid fuel: fundamental challenges and opportunities (a review). Renew Sust Energ Rev 31:221–257
175. Zhang LA, Chen QA et al (2011) Energy-saving direct ethanol production from viscosity reduction mash of sweet potato at very high gravity (VHG). Fuel Process Technol 91:1845–1850
176. Abdullah B, Muhammad SA et al (2019) Fourth generation biofuel: a review on risks and mitigation strategies. Renew Sust Energ Rev 107:37–50
177. Beacham TA, Sweet JB et al (2017) Large scale cultivation of genetically modified microalgae: a new era for environmental risk assessment. Algal Res 25:90–100
178. Adeniyi OM, Azimov U, Burluka A (2018) Algae biofuel: current status and future applications. Renew Sust Energ Rev 90:316–335
179. Hannon M, Gimpel J et al (2010) Biofuels from algae: challenges and potential. Biofuels 5: 763–784
180. Shinkawa S, Mitsuzawa S (2020) Feasibility study of on-site solid-state enzyme production by *Aspergillus oryzae*. Biotechnol Biofuels 13:1–15
181. Harris PV, Feng XF et al (2014) New enzyme in sights drive advances in commercial ethanol production. Curr Opin Chem Biol 19:162–170
182. Swami CS (2020) Studies on pectinase production in tomato fungi on different culture media. Ind J Appl Res 10(1):50–51
183. Yan S, Xu Y, Yu XW (2021) Rational engineering of xylanase hyper-producing system in *Trichoderma reesei* for efficient biomass degradation. Biotechnol Biofuels 14(1):1–7
184. Nagraj AK, Gokhale D (2018) Bacterial biofilm degradation using extracellular enzymes produced by *Penicillium janthinellum* EU2D-21 under submerged fermentation. Adv Microbiol 8:687–698
185. Ogunyewo OA, Randhawa A et al (2020) Engineered *Penicillium funiculosum* produces potent lignocellulolytic enzymes for saccharification of various pretreated biomasses. Process Biochem 92:49–60

186. Rastogi S, Soni R et al (2016) Unravelling the capability of *Pyrenophora phaeocomes* S-1 for the production of ligno-hemicellulolytic enzyme cocktail and simultaneous bio-delignification of rice straw for enhanced enzymatic saccharification. Bioresour Technol 222:458–469
187. Ahn YH, Yang YL, Choi CY (1995) Cellulases as an aid to enhance saccharification of cassava root. Biotechnol Lett 17:547–550
188. Riva S (2006) Laccases: blue enzymes for green chemistry. Trends Biotechnol 24:219–226
189. Singh A, Bajar S et al (2021) Evaluation of cellulase production from *Aspergillus niger* and *Aspergillus heteromorphus* under submerged and solid-state fermentation. J Environ Sustain 15:1–6
190. Gusakov AV, Salanovich TN et al (2007) Design of highly efficient cellulase mixtures for enzymatic hydrolysis of cellulose. Biotechnol Bioeng 97:1028–1038
191. Subramaniam Y, Masron TA (2020) The impact of economic globalization on biofuel in developing countries. Energy Convers Manag 10:100064
192. Li S, Tang B et al (2016) High-level production and characterization of laccase from a newly isolated fungus *Trametes* sp. LS-10C. Biocatal Agri Biotechnol 8:278–285
193. Corral OL, Villasenor-Ortega F (2006) Xylanases. In: Guevara-Gonzalez RG, Torres-Pacheco I (eds) Advances in agricultural and food biotechnology. Research Signpost, Kerala, pp 305–322
194. Beg QK, Kapoor M et al (2001) Microbial xylanases and their industrial applications:a review. Appl Microbiol Biotechnol 56:326–338
195. Haltrich D, Nidetzky B et al (1996) Production of fungal xylanases. Bioresour Technol 58: 137–161
196. Chauhan PS, Puri N et al (2012) Mannanases: microbial sources, production, properties and potential biotechnological applications. Appl Microbiol Biotechnol 93:1817–1830
197. Cai H, Shi P et al (2011) Acidicβ-mannanase from *Penicillium pinophilum* C1:cloning, characterization and assessment of its potential for animal feed application. J Biosci Bioeng 112:551–557
198. Duruksu G, Ozturk B et al (2009) Cloning, expression and characterization of endo-β-1,4-mannanase from *Aspergillus fumigates* in *Aspergillus sojae* and *Pichia pastoris*. Biotechnol Prog 25:271–276

Fungal Pectinases: Diversity and Multifarious Applications

15

D. C. Sharma, A. K. Mishra, and K. K. Mishra

Abstract

Enzymes are considered the backbone of green technology. Enzymes of microbial origin are being exploited in various industrial and environmental processes. They are used to degrade low-value polymers into valuable products or improve industrial processes. Pectin, the substrate of pectinase, is a polysaccharide usually found in the plant cell walls that acts as a cementing substance for binding the microfibrils of cellulose, hemicellulose, and protein. Structurally pectin is a diverse and complex polymer. Therefore, the enzymes involved in its degradation have evolved according to its structure and complexity. They are classified as pectin esterase, polygalacturonase, lyases, and protopectinase based on their mode of action. For industrial applications, they are classified as acidic and alkaline pectinases which find application in various industrial processes including fruit juice, tea, plant fiber retting, cotton fiber bioscouring, plant virus recovery, deinking of recycled paper, processing of pectin industry effluent from fruit juice industries and paper production, and a feed supplement. Due to their diverse nature and applications, various attempts have been made to optimize their production in submerged and solid-state fermentations in order to meet the industrial demand. Fungi are prime producers; therefore, the diversity, production, characterization, and applications are discussed.

Keywords

Pectin · Pectinase · Polygalacturonase · Alkaline pectinase · Submerged fermentation · Solid-state fermentation · Fungal pectinases · Fiber retting

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15.1 Introduction

Proteins that catalyze biochemical reactions without themselves being consumed in the process are known as enzymes. The exploitation of enzymes in industrial production gave rise to biotechnological processes having several advantages such as reduced production costs and lower energy consumption and pollution. Approximately 25,000 natural enzymes are estimated to be present in living beings; 25% of these are known and more than 120 of them are being used in various industrial applications. Enzymes play an increasingly important role in industrial processes. The global enzyme market was valued at US$ 10.69 in 2020 and is expected to grow at a compound annual growth rate (CAGR) of 6.5% from 2021 to 2028 (Grand View Research, 2021) [1]. The global food enzyme market reached a value of almost US$ a billion in the year 2020. The industry is further expected to grow at a CAGR of 5.3% between 2021 and 2026 and expected to reach a value of almost US$ 2.7 billion by 2026 (https://www.expertmarketresearch.com/reports/food-enzymes-market). The profitability of the industrial use of enzymes is particularly evident in the detergent, food and feed, and paper and textile industries (Fig. 15.1).

The Indian food enzyme market is projected to register a CAGR of 3.8% during the forecast period (2020–2025). The Indian market is growing at 7% per year and reached Rs. 2525crores for the last financial year. India comprises a large market for biotech-based products. Most of these products are produced by DSM, Novozymes, and Advanced Enzyme Technologies [2].

Microbes produce several pectin degrading enzymes, which play an essential role in the degradation of pectic substances, and have extensive applications in food processing, biological and organic degradation of plant materials, fermentation, and food spoilage. The major and fundamental industrial applications of microbial acidic pectic enzymes include extraction and clarification of fruit juices and in wine technology, maceration of vegetables and fruits, and the extraction of olive and citrus oils (Fig. 15.2). Alkaline pectinases find applications in fiber processing and treatment of wastewater from food processing industries for eliminating pectic substances.

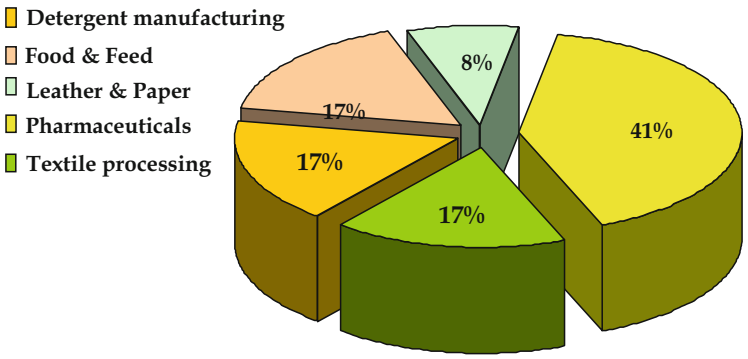

Fig. 15.1 Market share of enzymes in various industries

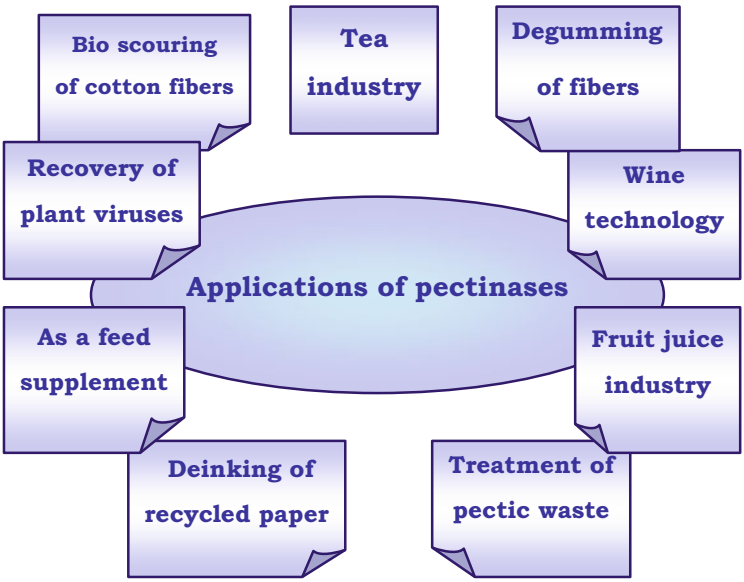

Fig. 15.2 Potential applications of pectinases in various industries

15.2 Pectins

Pectins, the major constituents of cereals, vegetables, fruits, and fibers, are complex high molecular weight, heterogeneous, and acidic structural polysaccharides. They are present in the middle lamella of plant cell wall as a thin layer. It acts as an adhesive extracellular material attached to cellulose microfibrils, surrounded by a matrix of hemicelluloses and proteins, acting as a "cementing" agent (Fig. 15.3a, b). Pectin is synthesized in Golgi apparatus as UDP-D-galacturonic acid during early stages of plant growth [3]. The pectic polysaccharides received attention due to their significant role in ripening of fruits and widespread application as a gelling agent in food processing industries. The presence of pectic material also influences the texture of fruits and vegetables; one of the most characteristic changes that occur during the ripening of fleshy fruits is softening that influences the texture and taste as well. In unripe fruits, pectin is bound to cellulose microfibrils in the cell walls. Pectin is an insoluble polysaccharide and, therefore, confers rigidity to the cell as well. These pectic substances are degraded and solubilized by naturally occurring enzymes in the fruits leading to softening of the fruits during the process of ripening [4]. During ripening, the neutral sugar composition of the pectin, however, does not change [5].

Pectin molecules consist of several hundred to thousands of D-galacturonic acid units that are linked together by α- (1,4)-glycosidic linkages in axial arrangement,

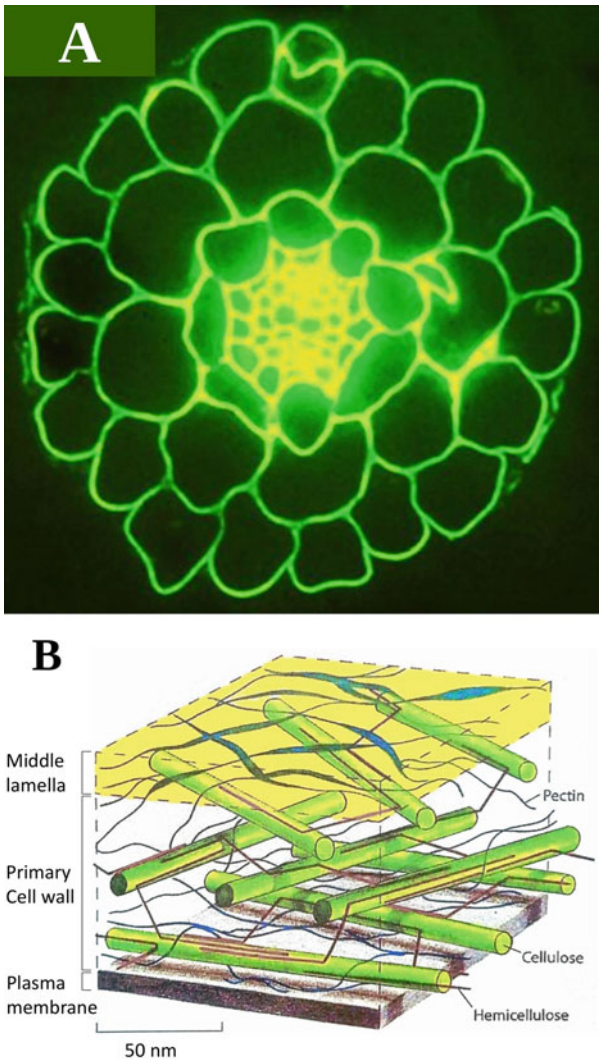

Fig. 15.3 Distribution and structure of pectin in plants. (**a**) Immunofluorescence micrographs showing the distribution of pectin epitopes in roots of *Arabidopsis* using monoclonal antibodies (transverse section). (**b**) Adhesive extracellular material (pectin) attached to cellulose microfibrils, surrounded by a matrix of hemicelluloses and proteins

which forms a *trans*-1, 4- polysaccharide, and it tends to coil around the screw axis [6] (Fig. 15.4). The carboxyl group of D-galacturonic acid unit in pectin is partially esterified with methanol or neutralized by monovalent or divalent cations like K^+, Na^+, and Ca^{2+}. Degree of esterification is 100% when the methoxyl content is 16.32%. Sometimes the hydroxyl groups at C_3 or C_4 positions get also acetylated, the degree of acetylation may vary from 0.18% - 2.5% [7]. The acetyl groups are so important because they determine the gelling properties of the polymer.

Additionally, rhamnose constitutes a small component of pectin backbone and is present as α-L-rhamnopyranose. They are present in various segments of structure:

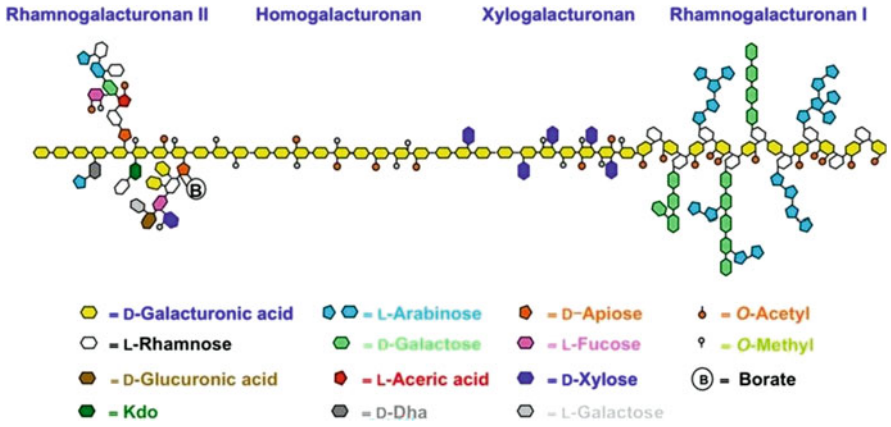

Fig. 15.4 Schematic structure of pectin

α-D-galactopyranosyluronicacid-(1 → 2)-α-L-rhamanopyranosyl-(1 → 4) galactopyranosyluronic acid [8–12]. The presence of rhamnose leads to the formation of a T-shaped 'kink' in the linear chain. Other various neutral sugars like L-arabinofuranose and D-galactopyranose occur most frequently in the side chains, while D-xylopyranose, D-glucopyranose, and L-fucopyranose are less common throughout the structure. D-Adipose, 2-O-methyl-D-xylose, and 2-O-methyl-L-fucose are widespread constituents of pectins present in minute quantities [11, 13]. These side chains are composed of neutral sugars that give the "hairy" character to rhamnogalacturonan portion of the pectin [5]. It is also observed that acidic and neutral pectins carry ferulic acid at non-reducing ends of the neutral arabinose and/or galactose-containing domains. It was suggested that they have an important role in the regulation of cell expansion as well as in lignification [14].

Pectic substances are soluble in water as well as in some organic compounds like formamide, ethylenediamine, and warm glycerol. The solubility of pectin molecule decreases in water as the length of polymer increases and vice versa. The esterification and acetylation of pectin polymer leads to increase in solubility in water. The aqueous solutions of pectic substances have high viscosity, which increases as the concentration of pectin increases and solubility decreases. The viscosity of pectin solution also increases as molecular weight and degree of esterification of pectic molecule increases. Parameters such as ionic strength, pH, and temperature of the solution also affect viscosity [4]. In cold, acidic solutions undergo de-esterification of the pectin polymer, while at higher temperatures, degradation of the polymer occurs. This property is generally exploited to get pectin with low methoxyl content. The treatment with strong acid results in a complete degradation that leads to the formation of CO_2, furfural, and many other products. In cold alkali, the saponification of methyl ester takes place, while at higher temperatures degradation of polymer occurs, which increases with increase in temperature. The degradation of pectin at

high temperature takes place through β-elimination of glycosidic bonds rather than hydrolysis, as occurs in case of other polymers, which makes it a unique polymer [15]. Heating of the polymer at neutral pH (pH 6–7) also results in the same pattern of its degradation. Variation in the degree of esterification leads to the alteration of its acidic and gelling properties. In completely esterified pectin, pH has no influence on the viscosity of solution, while in low esterified pectins, acidic pH (pH 4 or below) causes the association and hence the precipitation of polymer molecules. High energy radiations also cause degradation of pectin both in vivo and in vitro. The pectin polymer becomes selectively permeable for Ca^{+2} and other heavy metal ions on binding with insoluble polymers present in the cell. Although pectin is a hygroscopic polymer, its solubility decreases drastically when it absorbs moisture. Solubility increases when mixed with glycerol. Insoluble pectinates, cross-linked pectins, and protopectins show partial swelling in water. Chelating agents such as ethylenediaminetetraacetate (EDTA) increase the swelling of protopectin molecules by helping them in the removal of Ca^{+2} and other polyvalent cations [16].

Because of their most unique and outstanding physical properties, their ability to form thermo-reversible gels with sugar and acids, pectins are being used in jams and jellies for a long time. When sugar-free jellies are required, calcium ions are used to polymerize the pectinic acid. In gel formation, the cross-linking between the polysaccharide molecules results in the formation of regular twofold conformation and dimerization with specific inter-chain chelation of calcium ("egg box" binding). Each Ca^{+2} binds with an oxygen atom by nine coordinate bonds. Junction zones are formed mainly between the favorably arranged secondary hydroxyl groups of different macromolecules. The rate and temperature at which pectin gel formation occurs depend upon the degree of esterification with methanol. Ethyl and hydroxyl esters of pectic acids do not gel, whereas some amide groups favor the gelation process. Apart from food industry, pectin has lots of other applications like lubricants in food, especially in baby foods. Due to its colloidal properties, it acts as a lubricant and helps in the proper peristaltic movement in patients suffering from chronic constipation. It is also effective in the treatment of diarrhea and dysentery because the end products of enzymatic degradation of pectin have antimicrobial effect, which makes it effective. Due to its metal binding capacity, it can also be used as a detoxifying agent. It is also used as a drug carrier because it decreases the toxicity of the drug without affecting its potency. Pectin also has anti-cholesterol, antifibrinolytic, and hemostatic effects.

15.2.1 Nomenclature

In 1944, the Committee for Revision of Nomenclature of Pectic Substances, a former subdivision of American Chemical Society, defined pectic substances as those complex colloidal carbohydrate derivatives, which occur in plants and contain a large proportion of anhydro galacturonic acid units that are in a chain-like

combination. The carboxyl groups may be partially esterified by methyl groups and partially or completely neutralized by one or more bases.

The committee defined complex pectic substances as described below.

15.2.1.1 Protopectin

Protopectin is water-insoluble pectic substance observed in the cell wall of maximum plant tissues, besides ripening of fruits. On hydrolysis, it produces pectin and pectic acids. The insolubility of the protopectin depends on the polymer size and the presence of divalent cations like Ca^{2+}.

15.2.1.2 Pectinic Acids

These are the colloidal polygalacturonic acids with different proportions of methyl ester groups. Pectinates are normal or acidic salts of pectic acids; pectic acid alone has the unique property of forming a gel with sugars and acids or if it has reasonably low methyl content, with compounds such as calcium salts.

15.2.1.3 Pectin or Pectins

The pectin refers to the water-soluble polymeric material, which has various degrees of esterification with methanol, and can form gels with sugar and acid under appropriate conditions.

15.2.1.4 Pectic Acid

Pectic acid is a category designation used for the pectic substances mostly composed primarily of colloidal polygalacturonic acids and necessarily free of methyl ester groups. Pectic acids are mostly formed after tissue breakdown through the action of pectinmethylesterases. Normal or acidic salts of pectic acid are called pectates.

15.2.2 Biosynthesis of Pectic Substances

The plant cell wall surrounding the protoplast is a polysaccharide-rich complex, diverse, and dynamic entity of prime importance to plant growth and development [17]. Walls also have immense economic value, contributing to various agro-industrial processes and are the most important renewable energy source (biomass) on our planet [18]. Cell wall polysaccharides are synthesized by glycosyltransferases – most of which are located in the Golgi vesicles. Glycosyltransferases transfer the nucleotide sugar moiety to the acceptor substrate. Pectin is a very complex and heterogeneous structural polysaccharide.

15.2.3 Commercial Preparation of Pectin

The best raw materials for commercial pectin production are by-products of the fruit juice industries, i.e., apple pomace (dried) and citrus residues (peels) [19] (Table 15.1). Chemical extraction is the only way to extract pectin from these

Table 15.1 Pectin content of plant products

Source	Pectic substances (%)
Apple (fresh)	0.5–1.6
Bananas (fresh)	0.7–1.2
Carrots (dry matter)	6.9–18.6
Orange pulp (dry matter)	12.4–28.0
Sugar beet pulp (dry matter)	10.0–30.0

by-products. In chemical extraction, the raw materials are acid hydrolyzed in a pH range of 2.0–3.0 for 0.5–5 h at a temperature range of 70–100 °C in a liquid-to-solid ratio of 1:18. The pectin comes in liquid form and is separated by hydraulic press and/or centrifugation. The extract is then filtered again and finally concentrated to a standard setting strength. Preparation of powdered pectin involves treating concentrated liquid with organic solvents or certain metal salts to precipitate the polymer. This pectin precipitate is collected, dried, and ground [4].

15.3 Pectin-Degrading Enzymes

The enzymes responsible for breaking down pectin are widespread and have diverse modes of action. Pectinolytic enzymes include a group of enzymes that can catalyze the breakdown of pectin containing substrates [20] (Table 15.2). Based on their pH optima for activity, they can be categorized into acidic and alkaline pectinases.

15.3.1 Acidic Pectinases

The pectinases with pH optima below 7 are called acidic pectinases and find application in processes where reaction conditions are acidic, such as extraction and clarification of fruit juices, grape must, and wine making, maceration of vegetables and fruits, and the extraction of olive and citrus oils.

15.3.2 Alkaline Pectinases

The optimum pH for the activity of alkaline pectinases is above 7.0. Alkaline pectinases find applications in fiber processing and treatment of wastewater from food processing industries for eliminating pectic substances.

Table 15.2 Production of pectinases in different fermentation conditions and fermentation parameters

Microorganisms	Substrate Used	Fermentation Parameters			References
		Mode	Temp.	pH	
Aspergillus niger A 138	Sucrose	SmF	32	4.5	[60]
Aspergillus niger 3T5B8	Wheat bran	SSF	32	–	[61]
Penicillium veridicatum RFC3	Orange bagasse	SSF	30	–	[62]
Sporotrichum thermophile Apinis	Citrus pectin	SmF	45	7	[63]
Aspergillus fumigatus	Wheat bran	SSF	50	4-5	[64]
Aspergillus niger	Sunflower head	SSF	30	5.0	[65]
Aspergillus fumigatus MTCC 870	Wheat flour	SmF	30	5.0	[66]
Penicillium chrysogenum	Sucrose	SmF	35	6.5	[67]
Aspergillus heteromorphus	Orange peel	SmF	30	4.5	[68]
Thermomucor indicae-seudaticae	Wheat bran, Orange bagasse	SSF	45	–	[69]
Penicillium sp.	Pectin	SSF	35	6.0	[70]
Fomessclerodermeus	Soy and	SSF	28	–	[71]
Aspergillus niger	Pectin	SmF	37	5.5	[72]
Aspergillus sojae M3	Orange peel	SSF	22	–	[73]
Aspergillus flavus	Orange peel	SSF	40	5.5	[74]
Penicillium atrovenetum	Orange peel	SSF	40	5.0	[74]
Aspergillus oryzae	Orange peel	SSF	35	5.5	[74]
Pseudozyma sp. SPJ	Citrus peel	SSF	32	7.0	[75]
Mixed culture of *Aspergillus sydowii* *Aspergillus fumigatus*	Pineapple residue	SSF	35	5.0	[76]
Aspergillus niger	Sour orange peel	SSF	30	5.0	[77]
Penicillium citrinum	Sugar beet pulp	SSF	30	5.5	[78]
Aspergillus niger	Date pomace	SmF	–	6.18	[79]
Rhizomucorpusillus	Pectin	SSF	45	5.0	[80]
Rhodotorulaglutinis MP-10	Citrus pectin	SmF	30	5.5	[81]
Aspergillus sojae	Wheat bran	SSF	37	6.0	[82]
Aspergillus niger HFD5A-1	citrus pectin	SmF	30	4.5	[83]
Trichoderma viridi	orange peel	SSF	30	5.5	[84]
Aspergillus spp.	Citrus Pectin	SmF	30	4.5	[85]
Aspergillus niger strain MCAS2	Pectin	SmF	30	6.0	[86]
Aspergillus terreus NCFT4269.10	Banana peels	SSF	30	5	[87]
Aspergillus niger NRC1ami	Pectin	SmF	30	5.5	[88]

15.3.3 Type of Pectinases

15.3.3.1 Esterases

15.3.3.1.1 Pectinesterases (PMGE, EC:3.1.1.11)/Pectin Pectylhydrolase[SN]/Polymethylgalacturonate Esterase [RN]

Pectin esterases de-esterify the pectin molecule into pectic acid by attacking only the methyl ester group of pectic substances. The nucleophilic substitution leads to the formation of an intermediate acyl-enzyme complex with the release of methanol. This acyl-enzyme complex then undergoes deacylation, resulting in the regeneration of enzyme and pectic acid. The esterase preferentially acts block-wise on the methyl ester groups found adjacent to the free carboxyl groups in a sequential manner along the length of the polymer [4] (Fig.15.5). Some pectin esterases attack pectin only at the reducing end of the chain, while others attack the non-reducing end [21].

Kent et al. (2016) [22]studied the crystalline structure of pectin esterase of *Aspergillus niger* (Fig. 15.6). The high-resolution x-ray structures of pectinesterases from *Aspergillus niger* in deglycosylated and Asn-linked *N*-acetylglucosamine-stub forms reveal a $10^{2/3}$-turn parallel β-helix (similar to but with less extensive loops than bacterial, plant, and insect pectinesterases). Pectin esterases of microbial origin are not always alkaline proteins. They are active in the pH range between 4.5 and 9.0. The optimum pH for fungal pectin esterases is generally lower than that of bacteria [4]. The esterases of *Aspergillus oryzae* and *Trichoderma reesei* were optimally active at pH 8.5 and 7.5, respectively [23–25]. The de-esterification patterns of alkaline and acidic pectin esterases are different. The de-esterified pectin formed by the action of alkaline pectin esterase forms weak gels with Ca^{+2}, while the product of acid pectin esterase can form stronger gels with Ca^{2+} [26].

The temperature optima range between 25 and 55 °C. The esterase of *Myceliophthora thermophila* has the highest temperature optimum of 50 °C among fungi. The molecular mass of esterases ranges from 34,000 (*Aspergillus oryzae*) to 4,00,000 Da (*Clostridium multifermentans*). *E. chrysanthemi* esterase is a lipoprotein that undergoes side chain modification [27]. This is the most unstable esterase at room temperature as it loses all activity in 5 min, but retains its activity for more than a week when stored at 4 °C [24], an increase in the stability after immobilization of the enzyme. The enzyme is produced by many bacteria [28] and fungi [24, 28, 29].

The very popular and extensively utilized method for determining pectinesterase activity is the titrimetric method, in which the amount of carboxylic acid (-COOH)

Fig. 15.5 Reaction mechanism of pectinesterases

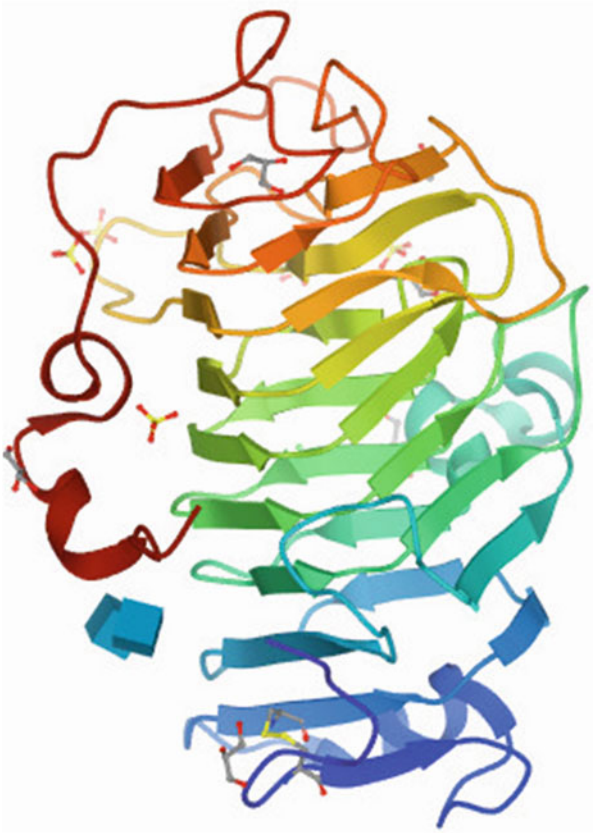

Fig. 15.6 Crystalline structure of pectin-esterases of *Aspergillus niger*

formed is quantified in terms of the alkali consumed to neutralize the reaction mixture. The enzyme activity can also be determined by measuring the amount of methanol formed by distillation and subsequent oxidation to formaldehyde as illustrated. Wood and Siddiqui (1971) [30] proposed a simplified spectrophotometric method by deleting the step of distillation of methanol. The most sensitive and accurate method is the conversion of methanol to methyl nitrite and its quantitation using HPLC [31].

15.3.3.2 Polygalacturonases (PG, EC 3. 2. 1. 15)/Poly (1, 4 - α- D - Galactosiduronate) Glycanohydrolase ^{SN}/Endopolygalacturonase^{RN}

Polygalacturonases cleave the glycosidic bonds of pectin by adding water molecules. They are generally considered acidic pectinases, which selectively attack α-1, 4-glycosidic linkages in pectic acid (polygalacturonic acid). There are only a few reports on alkaline endopolygalacturonases. The optimum pH for activity lies between 8.4 and 10.5.

15.3.3.3 Lyases

The lyases (transeliminases) form a group of pectinolytic enzymes that catalyze the breakdown of either pectic acid (polygalacturonate lyases) or pectin (polymethylgalacturonate lyases) by the β-elimination reaction. Two types of lyases are known.

15.3.3.3.1 Endopolygalacturonate Lyases (Endo-PGL, EC 4.2.2.2)/Poly (1, 4 - α- D - Galactosiduronate) Endolyase[SN]/Endopolygalacturonate Lyase (Endopectatelyase) [RN]

They are a group of hydrolytic enzymes, which randomly cleave α-1,4-glycosidic bonds by β-elimination in pectates and pectic acid, resulting in a rapid decrease in viscosity compared to the number of bonds broken (Fig.15.7). The pectate lyase from *Bacillus* sp. contained 353 residues and one calcium ion with α- and β-contents of 8.5 and 28.9, respectively (Fig. 15.8).

Endopolygalacturonate lyases are predominantly of microbial origin. Enzymes from the genera *Erwinia* and *Bacillus* are among the best known to cause soft-rot symptoms in plants (Perombelon and [32, 33]). The enzyme is also found in other microorganisms, including *Xanthomonas* [34], *Aspergillus* [35], and *Fusarium* [36], and finds application in fruit juice industry.

15.3.3.3.2 Exopolygalacturonate Lyases (Exo-PGL, EC 4.2.2.9)/Poly (1, 4 - α- D - Galactosiduronate) Exolyase[SN]/Exopolygalacturonate Lyase (Exopectatelyase) [RN]

Exopolygalacturonate preferentially targets pectates other than pectins, whereas polymethyl-galacturonate-methyl glycoside is unaffected. They release oligo-galacturonic acid from the reducing end of the substrate chain, and the smallest substrate that can be degraded is a trimer. At pH 8.0 to 9.5, they are optimally active. The complex of polygalacturonate lyase and pectin esterase works together on pectin, with the pectin chain moving directly from the esterase site to the polygalacturonate lyase site without dissociation or rebinding [37, 38]. The

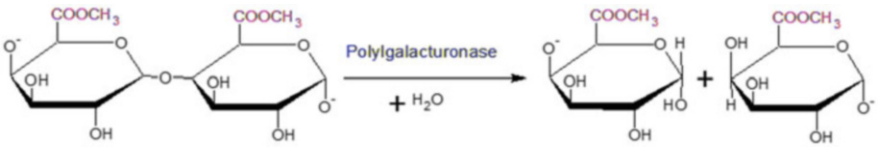

Fig. 15.7 Reaction mechanism of polygalacturonase

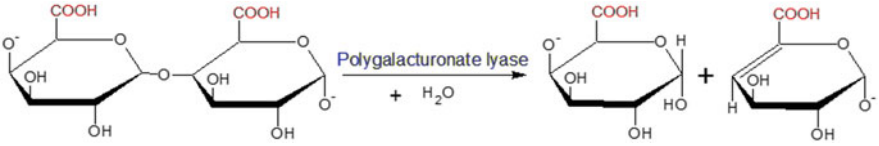

Fig. 15.8 Reaction mechanism of polygalacturonase lyase

polygalacturonate lyase can be released from pectin esterase by heating it [39]. Ca + 2 and Mn + 2, but not Mg2+ or Zn2+, are known to stabilize exopolygalacturonate lyases.

15.3.3.4 Poly-Methylgalacturonate Lyases (PMGL)

They are all endo-acting enzymes that catalyze the β-elimination between the fourth and fifth carbons of pectin at the non-reducing end, causing a quick drop in viscosity (Figs. 15.9 and 15.10). They are mostly of fungal origin and can degrade pectin directly; several species of *Aspergillus* and *Penicillium* are the rich sources of PMG, although they prefer highly esterified pectin as a substrate. They are most active when the pH is between 5.0 and 9.0. They do not require metal ions for their activity; however, depending on the pH and degree of esterification of pectin, a few authors have reported the need for Ca^{+2} and other cations [40, 41].

As the chain length of the substrate decreases, the activity of polymethyl galacturonate lyases falls rapidly. The enzyme of *Aspergillus fonsecaeus* degrades the smallest substrate, tetramethyl tetra galacturonate [42], and the enzyme of *Aspergillus niger* [43] degrades trimethyl-tri-galacturonate [42]. They are further divided into an endo- and exo-polymethyl galacturonate lyases based on their

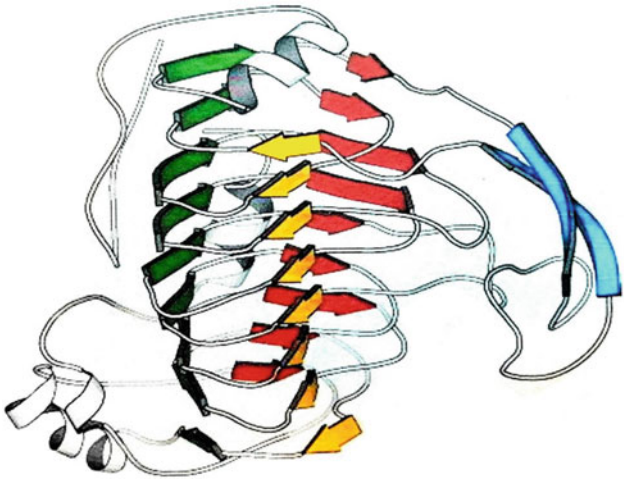

Fig. 15.9 Crystalline structure of pectin lyase from *Aspergillus* spp

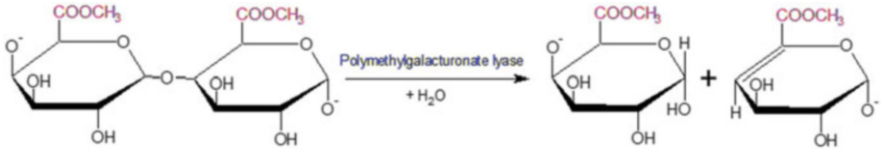

Fig. 15.10 Reaction mechanism of polymethylgalacturonate lyase

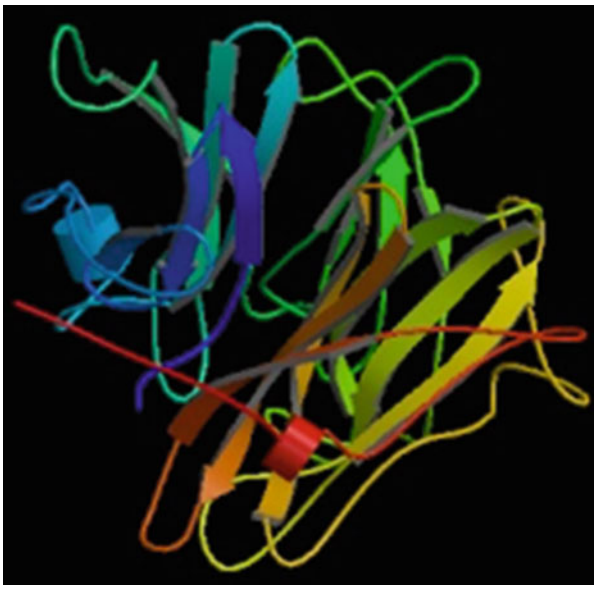

Fig. 15.11 Crystalline structure of protopectinase C (Arabinan endo-1,5-alpha-L-arabinosidase) of cellbriocellulosa

cleavage patterns. By trans-elimination, endo-polymethyl galacturonate lyases cause random cleavage in pectin molecules. While the exo-polymethyl galacturonate lyases cause sequential cleavage in pectin by a trans-elimination process.

15.3.3.5 Protopectinases (EC 3.2.1.99)

The insoluble protopectin is converted to highly polymerized water-soluble pectin by the action of a group of enzymes known as protopectinases (PPase). They are classified as A and B types depending on their mode of action. A-type protopectinases react with the smooth regions in the protopectin, which are composed of partially methoxylated galacturonic acid residues (Fig. 15.11). Protopectinases belonging to the B-type react with the hairy regions, which consist of rhamnogalacturonans and neutral sugar side chain, and thus, they are considered as glycan hydrolases (arabinases, galactanases) [19].

15.4 Production of Pectinolytic Enzymes by Microbes

The production of enzymes on a large scale is necessary for their utilization in related industries. Various fermentation processes are developed for their production and can be classified into two broad categories, i.e., submerged fermentation (SmF) and solid-state fermentation (SSF). Choice of fermentation method depends on the type of microbe and the focus of enzyme application. There is sufficient diversity of pectinase producers, and the enzyme produced by them has a broad range of pH and temperature optima. The type of microbe and the purpose of the enzyme application

influence the selection of the fermentation process. Pectinase producers are diverse, and the enzymes they produce have a wide pH and temperature ranges for activity. This could be due to the extensive distribution of polymers. Several microbes produce pectinases, including bacteria [30, 44–47],yeasts [48], and fungi [49–52]. Alkaline pectinases are primarily produced by bacteria, particularly of *Bacillus* species, whereas acidic pectinases are primarily produced by fungi and yeasts. Table 15.2 lists the most major fungal pectinase producers.

15.5 Optimization of Pectinase Production

Aguilar and Huitron (1990) [47]who optimized several aspects of pectinase production in submerged fermentation deserve credit for recent breakthroughs in pectinase production (SmF). Pectinase was produced in solid-state fermentation (SSF) by Lonsane et al. (1992) [53]. Liao et al., (1988) [54] evaluated pectinase yield and productivity in SSF and SmF. Differences in enzyme profiles and kinetics of pectinases generated in SSF and SmF were reported by Acuna-Arguelles et al. (1995) [55].

15.5.1 Enzyme Production in Submerged Fermentation

15.5.1.1 Effect of Carbon Sources

Carbon is well-known to be a primary limiting factor in cellular metabolism and enzyme production. Pectin has been utilized as the sole source of carbon for pectinase synthesis in various studies. Pectinase production is induced by polygalacturonic acid or pectin, which then serves as a carbon source when reacted upon by pectinases. Sugars in addition to corn powder have been discovered to be optimal for *Aspergillus* strains to synthesize pectinase [56]. The influence of carbon source on *A. niger* CH4 pectinase synthesis revealed that high sugar concentrations increased pectinase production in SSF, but pectinase production declined inversely with sugar concentration in SmF. The observation is identical to the previous one [57, 58]. A broad range of carbon sources was used for pectinase production in SmF and SSF are summarized in Table 15.2.

15.5.1.1.1 Effect of Nitrogen Sources

Organic and inorganic nitrogen sources are the most commonly used nitrogen sources. Yeast extract is the most extensively utilized organic nitrogen source. It's also been suggested that it acts as a pectinase inducer [59]. For the synthesis of pectinolytic enzymes, yeast extract was combined with organic and inorganic nitrogen sources.

15.5.1.1.2 Effect of Initial pH of the Medium

The best pH for pectinase synthesis varies depending on the microbial strain. In the pH range of 7.0 to 10.0, enzyme production was high [16, 43, 44, 46, 59, 89]. Acidic

pH (4.0–6.8) favored enzyme production in a few fungi, such as *Aspergillus* spp. and *Penicillium* spp. (Table 15.2).

15.5.1.1.3 Effect of Incubation Temperature

At 30 °C or less, the majority of bacteria and fungi produce pectinases. At 22 °C, *Aspergillus sojae* M3 produced pectinase, according to Demir et al. (2011) [90]. At 40, 45, and 50 °C, *Aspergillus flavus* [74], *Thermomucor indicae-seudaticae* [69], and *Aspergillus fumigatus* [64]were found to produce the enzyme optimally.

15.5.1.1.4 Effect of Inducers on Pectinolytic Enzyme

Pectinase production is inherent in certain organisms but inducible in others. In certain fungal species, constitutive polygalacturonic acid transaminase synthesis has been detected, while catabolite suppression has been found in the presence of excess substrate [34]. On varied carbon sources, *Aspergillus* sp. CH-Y-1043 elaborated exopectinase constitutively [47]. Pectin was the most effective inducer in species with inducible enzyme systems, followed by polygalacturonic acid [91].

15.5.1.1.5 Effect of Cations

Metal ions are crucial in the production and activation of a variety of extracellular enzymes. Ca2+ is the most significant metal ion in pectinase synthesis among all metal ions. In the absence of Ca2+, Kelly and Fogarty (1978) [16]found that polygalacturonase lyase was produced, but that the level of production rose dramatically when Ca2+ levels increased. It's also been hypothesized that Ca2+ has a function in enzyme activation [43]. Other monovalent and divalent cations, alone or in combination with Ca2+, have been demonstrated to suppress pectinase synthesis. EDTA reduced the synthesis of pectinase [16].

15.5.1.2 Solid-State Fermentation (SSF)

Solid-state fermentation exploits inert substrates as solid support in the absence or near absence of free-flowing water [92]. In recent years, solid-state fermentation has gotten a lot of attention for producing various bioactive compounds [49, 93]. This procedure is particularly profitable for countries with a lot of biomass and agro-industrial waste. The SSF provides the following benefits over submerged fermentation [94–96]:

(a) Low water requirement.
(b) Cheap media for the fermentation.
(c) Less stringent requirement of aseptic conditions due to the inability of most contaminants to grow in the absence of free-flowing water.
(d) Use of high concentration of substrate.
(e) Higher product yields.

Wheat bran, rice bran, sugarcane bagasse, corncobs, and apple pomace are among the best substrates for the SSF [97][Table 15.3]. There have been reports of pectinase production from wheat bran [98], citrus pulp-pellets [99], lemon and orange peel

Table 15.3 Different agro-residues used for solid-state cultivation of fungi and bacteria

Starchy	Cellulosic	Pectin	Oily
Wheat bran	Wheat straw	Citrus pulp	Mustard oil seed cake
Rice	Rice Stover	Citrus peel	Cotton oil seed cake
Cassava	Corn Stover	Apple pulp	Linseed cake
Corn	Sugar beet pulp	Sun hemp	
Banana meal	Feedlot waste	Peer pulp	
Buckwheat			
Seeds wood			
Rye meal			

[100, 101], coffee pulp [102], sugar beet pulp [103], and sugarcane bagasse [49]. Particle size and moisture level/water activity (a_w) are critical parameters that influence microbial growth and enzyme production on a given substrate [104].The synthesis of polygalacturonase decreased as the substrate's water activity decreased [105]. A significant titer of pectinase was reported in SSF in a comparative investigation of *Aspergillus* spp. pectinase synthesis in SmF and SSF [106]. *A. niger* pectinase produced in SSF was more thermotolerant and active over a wider pH range than that produced in SmF [49].

15.6 Characteristics of Purified Pectinases

Knowledge of the features of microbial pectinases is required for investigating the application of enzymes in wide range of industrial processes. As a result, many studies have been focused on pectinases stability, reaction conditions, and catalytic performance. The pH and temperature optima, as well as thermal stability, are the properties that cause deactivation and stability and are, therefore, regarded as key barriers to the rapid development of biotechnological processes. Enhancing the stability of enzymes and maintaining the required level of activity over time are two significant factors to consider when choosing enzymes. The stability of enzymes is determined by both physical (pH and temperature) and chemical (inhibitors and activators) characteristics. The biochemical features of various pectinases are summarized in Table 15.4.

Heikinheimo [28, 107–109] found that the ideal pH for alkaline pectinase was between 7.5 and 11.5. The pH and temperature used in the experiment are not optimal for stability and production, which is consistent with the fact that pectinase catalytic performance (activity) and stability are quite different [110]. Deactivation is frequently used as the first-order reaction when calculating thermodynamic parameters. Both partially purified and unprocessed enzymes have a negative deactivation entropy. This could be due to structural effects (enzyme unfolding) or the surrounding environment.

Chemical modifications have been used to try to stabilize pectinases. On two separate types of *Aspergillus* PG, Kuchenreuther and colleagues (2009) [111] used

Table 15.4 Properties of various purified pectinases of fungal origin

Source of Enzyme	Nature	Molecular Wt. (kDa)	pI	K_m	Opt. Temp °C	Opt. pH	Temp. stability	pH stability	Reference
Polygalacturonases									
Aspergillus japonicus	Endo	38 (PG I)	5.6	–	30	4.0–5.5	–	–	115
	Endo	65 (PG II)	3.3	–	30	4.0–5.5	–	–	
Mucorflavus –	40	8.3	–	45	3.5–5.5	40	2.5–6.0	116	
Sclerotinia borealis	Endo	40	7.5	-	40–50	5.0	50	3.5–5.5	117
Aspergillus niger	Endo	61 (PG I)	–	0.12	43	3.8–4.3	50	–	118
	Endo	38 (PG II)	–	0.72	45	3.0–4.6	51	–	
Penicillium frequentans	Exo	63	–	1.6	50	5.0	–	–	119
	Exo	79	–	0.059	50	5.8	–	–	
Aspergillus awamori	Endo	41	6.1	–	40	5.0	50	4.0–6.0	120
Sclerotinia sclerotiorum	Endo	42 (PG8b)	4.8	0.8	50	4.0	–	–	121
	Endo	41.5 (PG8u)	4.8	0.5	50	3.5	–	–	
Stereum purpureum	Endo	42 (PG1a)	8.5	–	–	5.0	75	–	122
	Endo	44 (PG1b)	8.5	–	–	5.0	75	–	
	Endo	46(PG1c)	8.5	–	–	5.0	75	–	
Saccharomyces pastorianus	–	43	5.4	–	50	4.2	–	–	123
Saccharomyces cerevisiae IMI-8b	–	43	–	–	45	4.5	–	–	124
Fusarium oxysporum f. sp. Lycopersci	Exo	38	–	–	69	11.0	–	7.0–11.0	125
Saccharomyces cerevisiae	Endo	39	–	4.7	45	5.5	50	4.5–6.0	126
Kluyveromycesmarxianus	Endo	496 (PG I)	6.3	–	–	–	–	127	
	Endo	496 (PG II)	6.0	–	–	–	–	–	
	Endo	496 (PG III)	6.3	–	–	–	–	–	
	Endo	496 (PG IV)	5.7	–	–	–	–	–	
Aspergillus niger	–	–	–	0.367	40	5	–	–	128
Aspergillus fumigatus	–	–	–	0.156	40	5	–	–	
Aspergillus flavus	–	–	–	0.261	45	5	–	–	
Pectin lyases									
Penicillium italicum	PMGL	22	8.6	3.2	40	6.0–7.0	50	8.0	129
Aspergillus japonicus	PMGL	–	7.7	0.16	55	6.0	–	–	130
Penicillium adametzii	PMGL	–	–	–	60	8.0	40	7.0	131
P. citrinum	PMGL	–	–	–	45	7.0	40	7.0	
P. janthinellum	PMGL	–	–	–	40	6.5	40	7.0	
Amycolata sp.	PGL	31	10.0	0.019	70	10.3	50	6.0–8.0	132
Pythium splendens	PMGL	23	8.0	–	–	8.0	50	–	133
Pichia pinus PMGL	90	–	0.33	–	4.5	50	–	134	
Thermoascusauratniacus PMGL	–	–	–	65	10.5–11.0	70	4.0	123	
Fusarium monoliforme	PGL	–	–	–	–	8.5	75	–	135
Pectinesterases									
Aspergillus niger	-	-	1.01	45	5.0	-	-	-	136,
Aspergillus japonicus	46 (PE I)	3.8	-	-	4.0-5.5	50	-	-	115
	47(PE II)	3.8	-	-	4.0-5.5	50	-		

various tyrosine reactive agents such as acetic anhydride, N-acetyl imidazole, and tetranitromethane. Metal ions are required for pectin methylesterase activity due to the presence of carboxyl groups on pectin, which may trap enzyme molecules and prevent the enzyme cation from interacting with carboxyl groups, allowing the enzyme to engage with the ester bonds to be cleaved. Similarly, excessive metal

ion concentrations cause enzyme inhibition due to the blockage of carboxyl groups present next to the ester bond to be cleaved. The enzyme is unable to recognize the location of action if these groups are inhibited by metal ions [112].

15.7 Cloning of Pectinase Encoding Genes

There are only a few papers on pectinase cloning and characterization. Guo et al. [113] isolated pectate lyase gene (*pelC*, 1.3-kb) from *Fusarium solani pisi* using *pelB* cDNA as a probe. The coding region of *pelB* was amplified by reverse transcription-polymerase chain reaction using total RNA isolated from a pectin-induced *F. solani pisi* culture as template. The open reading frame of *pel*C was predicted to encode a 23.3-kDa protein of 219 amino acid residues, which shared 51% identity with PLA from *F. solani pisi*. The amplified *pel*C cDNA was expressed in *Pichia pastoris* that expressed a pectate lyase C (PLC) with a molecular mass of 26.0 kDa that contained carbohydrate. The purified PLC required Ca^{2+} for its activity and exhibited optimal lyase activity at pH 9.5 and 55 °C.

Guo et al. (1995) [113] used *pelA* cDNA as a probe to isolate the pectate lyase gene (*pel*C, 1.3-kb) from *Fusarium solani pisi*. Using total RNA obtained from a pectin-induced *F. solani pisi* culture as a template, the coding area of *pel*C was amplified by reverse transcription-polymerase chain reaction. *Pel*C's open reading frame was predicted to encode a 23.3-kDa protein with 219 amino acid residues, which shared 51% similarity with PLA from *F. solani pisi*. The amplified *pel*C cDNA was expressed in *Pichia pastoris*, which produced pectate lyase C (PLC) with a molecular mass of 26.0 kDa that was a glycoprotein. The pure PLC needed Ca^{2+} to function, and its lyase activity was best at pH 9.5 and 55 °C. A western blot employing antibodies produced against both PLA and PLC. Both PLC and PLA are immunologically related to each other.

15.8 Applications of Pectinases

Degumming and retting of plant fibers, bioscouring of cotton fibers, recovery of plant viruses, tea business, deinking of recycled paper, processing of pectic effluent from fruit juice industries, paper production, and as a feed supplement are all applications for alkaline pectinases. Plant fibers are dead sclerenchymatous cells that are long, narrow, thick walled, and lignified. They perform a strictly mechanical role of providing support and stiffness to the plant body [135].

Plant fibers are divided into three categories based on their origin and structure: (a) soft or bast fibers, which are formed in groups outside of xylem in the cortex, phloem, or pericycle, e.g., Ramie (*Boehmeria nivea*) and sunn hemp (*Crotalaria juncea*); (b) surface fibers, which are produced on the surface of stems and leaves, e.g., cotton (*Gossypium* spp.); and (c) hard or structural fibers that are supportive and conducting fibrovascular bundles chiefly found in monocots, e.g., Manila hemp (*Musa textilis*).

Pectin makes up more than 40% of the dry weight of plant cambium cells, according to Bajpai (1999) [136]. These fibers must be cleared of the sticky material before being used in industrial applications. Degumming and retting are terms that describe the process of removing non-cellulosic sticky material from plant fiber. Mechanical processing and treatment of fiber with 12–20% NaOH (2% NaOH in the case of sun hemp and ramie fiber) coupled with wetting and reducing agents could eliminate the gummy material. This is inefficient because the gummy material of the fiber is not entirely removed, and cellulose is removed in a non-specific manner, resulting in a significant weight loss of fiber. Because of this disadvantage, fiber treatment with an alkaline solution containing pectinases is preferable [137, 138].

Retting of jute, flax, hemp, ramie, kenaff (*Hibiscus sativa*), and coir from coconut husks also involves action of pectinolytic enzymes and microorganisms [50, 139]. Certain bacteria (e.g., *Clostridium*, *Bacillus)* and fungus (e.g., *Aspergillus*, *Penicillium*) have been found to degrade the bark's pectin and release fiber [140]. In an open field, biological retting can be accomplished by spreading plant straw on the ground and exposing it to the action of fungi for 2–10 weeks. This is termed as dew retting (anaerobic process).

The species of *Aspergillus*, *Penicillium*, *Rhodotorula*, and *Cladosporium* have been isolated from dew-rotted plants [141]. Due to the presence of cellulolytic bacteria, the technique does not produce high-quality fiber. The retting process can also be done in closed water tanks or water containing anaerobic bacteria [139]. The tank and water pits quickly run out of dissolving oxygen, promoting the growth of anaerobic microbial flora [142], and have been shown to macerate straw fiber in the laboratory. Pectinases, which are of fungal origin, cause fiber separation during flax retting [143]. Fungi in particular play a significant role in retting processes of the flax in addition to fiber bleaching and removal of waxes and gums [144].

15.8.1 Ramie (*Boehmeria Nivea*) Bast Fibers

Ramie bubblegum is found in 20–35 percent of ramie fibers. The fiber can be used in the textile industry when the gum (pectin and hemicellulose) is removed. As noted previously, ramie fiber is degummed using chemical methods. This procedure not only consumes a lot of energy but also pollutes the environment significantly. A combination approach was proposed to tackle this challenge, in which microorganisms and their enzymes carry out the process at low temperatures without the use of chemical agents [145].

Cao et al. (1992) [146]described degumming fibers with neutrophilic bacteria, but these strains have not been used on an industrial scale due to challenges in preventing heterogeneous contamination and other drawbacks. The search for new microorganisms and enzymes for such an application is therefore still a challenging task. Several fungi have been identified to be part of the fiber-deteriorating microflora of ramie. These organisms including *Sclerotium* sp., *Aspergillus fumigatus*, *Fusarium oxysporum*, and *Penicillium* sp. could play a beneficial role in degumming

of ramie fibers. The degumming process is accelerated when decorticated ramie fiber is pretreated with alkali [50].

15.8.1.1 Buel (*Grewia Optiva*) Bast Fibers

Dumping the stems in stagnant water for months removes the bast fibers. Retting is aided by the natural microflora on the fibers. Physical separation of the fibers is then performed. Due to the attack of cellulolytic microbes during the prolonged incubation, the quality of the fibers obtained this way was poor.

15.8.1.2 Pretreatment of Pectic Wastewater

Pectinaceous compounds are abundant in the wastewater from the citrus processing sector. Pectic wastes are not entirely digested by the naturally occurring bacteria present in activated sludge during treatment, causing filter obstruction [147]. For the treatment of wastewater from citrus processing companies, many technologies such as physical dewatering, spray irrigation, chemical coagulation, direct activated sludge treatment, and chemical hydrolysis followed by methane fermentation are available [147]. All procedures have some limitations, such as reduced efficiency due to pectic material chemical resistance, high treatment costs, long treatment times, and process complexity. Tanabe et al. [148] discovered an endopectate lyase-producing alkaliphilic *Bacillus* sp. (GIR 621) (optimal pH for growth 10) for pectic wastewater treatment.

Erwinia carotovora (FERM P- 7576), a well-known plant pathogen that secretes endo-pectate lyase and is useful in the pretreatment of pectinaceous wastewater, has been discovered to be useful in the pretreatment of pectinaceous wastewater [148]. It cannot be utilized directly due to its strong phytopathogenecity. A bacterium-produced pectolytic enzyme was reported to solubilize practically all of the pectic compounds found in wastewater in an indirect pretreatment method.

15.8.1.3 Production of Japanese Paper

Mitsumata bast fibers are retted to create Japanese paper [149]. Bast fibers are traditionally processed via the soda-ash frying procedure. Due to its significant macerating action, alkaline pectinase generated by *Aspergillus niger* is used in an alternative technique [86, 148]. These retted bast fibers were employed in the making of Japanese paper. The pulp obtained through bacterial retting had the same strength as that obtained through the traditional soda-ash heating process. The uniformity and softness of the paper sheets made from this pulp were impressive.

15.8.1.4 Paper Making

Enzymes are becoming widely used in the paper industry as they transform the substrate of interest in the presence of other chemically related molecules [150]. In mechanical pulps bleached with hydrogen peroxide, pectinase is used to overcome retention difficulties. The continuous filtration process in papermaking converts dilute slurry of fibers, fiber fragments (fines), and inorganic filler particles (clay or CaCO3) into sheets. The water must be emptied regularly. A filter cloth with pores is

used for this, allowing fiber fragments and filler particles to flow through. Some drainage mechanisms are employed to keep the "fine" and "filler" in the paper sheets in place, allowing for faster water drainage. Various cationic polymers with varied architectures are routinely employed as retention aids [151]. Bleaching pulps with alkaline peroxide solubilizes the polysaccharides present in the pulp, the most notable of which are pectins or polygalacturonic acid, which are interfering compounds [150, 152]. Polygalacturonic acids have a propensity for forming gels with cationic polymers (cationic demand). Cationic demand is highly influenced by the degree of polymerization; monomers, dimers, and trimers have little cationic demand, whereas hexamers and long chains have a lot [153]. The retention devices choke as a result of this. To lower the cationic demand of pectin solutions from peroxide bleaching, pectinase is employed to degrade the polysaccharide into monomers, dimers, and trimers [154].

15.8.1.5 Application in Coffee and Tea Fermentations

By dissolving the pectins, pectinase treatment promotes tea fermentation while also reducing the foam-forming ability of instant tea granules [155]. Pectinases are sometimes used to remove the pectic substance-rich pulpy bean layer. The ability of cellulose and hemicellulase enzyme preparations to aid in the digestion of mucilage has also been explored [156, 157]. Fungal pectinases have also been reported to be employed in the production of tea.

15.8.1.6 Recovery of Plant Viruses

Because of the presence of cellulose and pectin in the phloem, recovering plant viruses is difficult. Under these situations, alkaline pectinase and cellulase have been utilized to recover viruses [83].

15.8.1.7 Recovery of Plant DNA

Contaminants combine with DNA or RNA to form a co-precipitate. To overcome this difficulty, Renuka et al. (2009) [158]suggested using alkaline pectinase in the last DNA-hydration phase.

15.9 Industrial Production and Commercial Suppliers of Pectinases

Most of the commercially produced pectinases are of fungal origin, mainly from the genus *Aspergillus.* Enzymes produced by the strains *A. niger*, *A. oryzae*, *A. wentii*, and *A. flavus* successfully met the requirements of fruit juice industries and thus have been exploited. The combination of pectin esterase polygalacturonase and polygalacturonate lyase is the most suited, and as a result, most commercial formulations contain a mixture of these in varying ratios [159]. In the commercial production of pectinase, a variety of solid-state and submerged fermentation processes are utilized, the most prevalent of which are surface bran culture (Koji method), deep tank (submerged method), and two stages submerged fermentation.

The substrate is saturated with the mineral solution until it absorbs all of the water and then injected with fungus spores using the Koji method. The enzyme is restored in 1–6 days. Due to the absorption of the enzyme, recovery of the enzyme is a bit difficult in this process.

For the solid-state synthesis of the enzyme, Rombouts and Pilnik (1980) [160] reported an alternative approach (rotary drum). The enzyme can be produced by using submerged fermentation (SmF). Enzyme is available in two forms: liquid and powder. More than 15 companies provide pectinases [161].

References

1. Enzymes Market Size & Share Report, 2021–2028. (n.d.). Market Research Reports & Consulting | Grand View Research, Inc. Retrieved January 19, 2022, from https://www.grandviewresearch.com/industry-analysis/enzymes-industry
2. Sakai T, Sakamoto T et al (1993) Pectin, pectinase and protopectinase: production, properties, and applications. Adv Appl Microbiol 39:213–294
3. De Vries C, De Jager C et al (1987) Nuclear charge-density-distribution parameters from elastic electron scattering. At Data Nucl Data Tables 36(3):495–536
4. Whistler RL, Smart CL (1953) Isolation of crystalline D-glucose and cellobiose from an enzymatic Hydrolyzate of Cellulose1. J Am Chem Soc 8:1916–1918
5. McComb EA, McCready RM (1957) Determination of acetyl in pectin and in acetylated carbohydrate polymers. Anal Chem 5:819–821
6. Aspinall GO, Craig JWT, Whyte JL (1968) Lemon-peel pectin. Carbohydr Res 4:442–452
7. Aspinall GO, Gestetner B et al (1968b) Pectic substances from lucerne (Medicago sativa). Part II. Acidic oligosaccharides from partial hydrolysis of leaf and stem pectic acids. Journal of the Chemical Society C: Organic 2554
8. Aspinall GO, Craig JWT et al (1970) Lemon-peel pectin. Carbohydr Res 4:442–452
9. Aspinall D, Paleg LG (1981) Proline accumulation: physiological aspects. In: Paleg LG, Aspinall D (eds) The physiology and biochemistry of drought resistance in plants. Academic Press, Sydney, pp 205–241
10. Lau RR (1985) Two explanations for negativity effects in political behavior. Am J Polit Sci 1: 119
11. Darvill AG, McNeil M, Albersheim P (1978) Structure of plant cell walls. Plant Physiol 3: 418–422
12. Reshmy R, Philip E et al (2021) Nanobiocatalysts: advancements and applications in enzyme technology. Bioresour Technol 337:125491
13. Díaz AB, Alvarado O, de Ory I, Caro I, Blandino A (2013) Valorization of grape pomace and orange peels: improved production of hydrolytic enzymes for the clarification of orange juice. Food Bioprod Process 91(4):580–586
14. Deuel H, Stutz E (1958) Pectic substances and pectic enzymes. Adv Enzymol Relat Subj Biochem 20:341–382
15. Trentini MMS, Toniazzo G et al (2015) Purification of pectinases from aspergillus Niger ATCC 9642 by ethanol precipitation. Biocatal Agric Biotechnol 3:315–320
16. Kelly CT, Fogarty WM (1978) Production and properties of polygalacturonate lyase by an alkalophilic microorganism bacillus sp. RK9. Can J Microbiol 10:1164–1172
17. Somerville C, Bauer S et al (2004) Toward a systems approach to understanding plant cell walls. Science 5705:2206–2211
18. Bacic ILZ, Rossiter DG, Bregt AK (2006) Using spatial information to improve collective understanding of shared environmental problems at watershed level. Landsc Urban Plan 1–2: 54–66

19. Esquivel Bazan E, Garcia Barrios LE et al (1999) Forage legumes: a resource for sustainable agricultural production in Chamula. Chiapas Agrociencia 4:501–507
20. Loera O, Aguirre J, Viniegra-González G (1999) Pectinase production by a diploid construct from two aspergillus Niger overproducing mutants. Enzym Microb Technol 1–2:103–108
21. Miller L, Macmillan JD (1970) Mode of action of pectic enzymes II. Further purification of exopolygalacturonate lyase and Pectinesterase from *clostridium multifermentans*. J Bacteriol 1:72–78
22. Kent LM, Loo TS, Melton LD et al (2016) Structure and properties of a non-processive, salt-requiring, and acidophilic pectin Methylesterase from *aspergillus Niger* provide insights into the key determinants of processivity control. J Biol Chem 291(3):1289–1306
23. Aguilar AF et al (2017) Re-evaluation of pectin (E 440i) and amidated pectin (E 440ii) as food additives. EFSA J 7
24. Markovič O, Biely P, Mislovičová D (1985) Sensitive detection of endo-1,4-β-glucanases and endo-1,4-β-xylanases in gels. Anal Biochem 1:147–151
25. Kazemi-Pour N, Condemine G et al (2004) The secretome of the plant pathogenic bacterium *Erwinia chrysanthemi*. Proteomics 10:3177–3186
26. Li D, Dong H et al (2021) Pectin in biomedical and drug delivery applications: a review. Int J Biol Macromol:49–65
27. Wai WW, Alkarkhi AFM, Easa AM (2010) Effect of extraction conditions on yield and degree of esterification of durian rind pectin: an experimental design. Food Bioprod Process 2–3:209–214
28. Shevchik VE, Hugouvieux-Cotte-Pattat N (1997a) Identification of a bacterial pectin acetyl esterase in Erwinia chrysanthemi 3937. Mol Microbiol 6:1285–1301
29. Maldonado MC, Strasser de Saad AM (1998) Production of pectinesterase and polygalacturonase by aspergillus Niger in submerged and solid-state systems. J Ind Microbiol Biotechnol 1:34–38
30. Wood PJ, Siddiqui IR (1971) Determination of methanol and its application to measurement of pectin ester content and pectin methyl esterase activity. Anal Biochem 2:418–428
31. Bartolome LG, Hoff JE (1972) Firming of potatoes. Biochemical effects of preheating. J Agric Food Chem 2:266–270
32. Kelman A, Perombelon MCM (1980) Ecology of the soft rot Erwinias. Annu Rev Phytopathol 1:361–387
33. Collmer A, Keen NT (1986) The role of pectic enzymes in plant pathogenesis. Annu Rev Phytopathol 1:383–409
34. Vaughan ILH, Jakubczly T et al (1969) Some pink yeasts are associated with softening of olives. Appl Microbiol 18:771–775
35. Nasuno S, Starr M (1967) Polygalacturonic acid trans-eliminase of Xanthomonas campestris. Biochem J 1:178–185
36. Dean RA, Timberlake WE (1989) Production of Cell Wall-degrading enzymes by aspergillus nidulans: a model system for fungal pathogenesis of plants. Plant Cell 3:265
37. Crawford MS, Kolattukudy PE (1987) Pectate lyase from *fusarium solani* f. sp. pisi: purification, characterization, in vitro translation of the mRNA, and involvement in pathogenicity. Arch Biochem Biophys 1:196–205
38. Sato M, Kaji A (1980) Exopolygalacturonate lyase produced by *streptomyces massasporeus*. Agric Biol Chem 4:717–721
39. Sheiman M, Macmillan I et al (1976) Coordinated action of Pectinesterase and polygalacturonate lyase complex of *clostridium multifermentans*. Eur J Biochem 2:565–572
40. Edstrom RD, Phaff HJ (1964) Eliminative cleavage of pectin and of oligogalacturonide methyl esters by pectin trans-Eliminase. J Biol Chem 8:2409–2415
41. Ishii S, Yokotsuka T (1973) Susceptibility of fruit juice to enzymic clarification by pectin lyase and its relation to pectin in fruit juice. J Agric Food Chem 2:269–272
42. Satapathy S, Rout JR et al (2020) Biochemical prospects of various microbial pectinase and pectin: an approachable concept in pharmaceutical bioprocessing. Front Nutr 7:117–122

43. Horikoshi K (1972) Production of alkaline enzymes by alkalophilic microorganisms part III. Agric Biol Chem 2:285–293
44. Karbassi A, Vaughn RH (1980) Purification and properties of polygalacturonic acid trans-eliminase from *Bacillus stearothermophilus*. Can J Microbiol 3:377–384
45. Sakellaris G, Nikolaropoulos S, Evangelopoulos AE (1989) Purification and characterization of an extracellular polygalacturonase from *lactobacillus plantarum* strain BA 11. J Appl Bacteriol 1:77–85
46. Singh Dosanjh N, Hoondal GS (1996) Production of constitutive, thermostable, hyper active exo-pectinase from bacillus GK-8. Biotechnol Lett 12:1435–1438
47. Aguilar G, Huitron C (1990) Constitutive exo-pectinase produced by *aspergillus* sp. CH-Y-1043 on different carbon source. Biotechnol Lett 9:655–660
48. Borin MF, Said S, Fonseca MJV (1996) Purification and biochemical characterization of an extracellular Endopolygalacturonase from penicillium frequentans. J Agric Food Chem 6: 1616–1620
49. Solis-Pereyra S, Favela-Torres E et al (1996) Production of pectinases by aspergillus Niger in solid-state fermentation at high initial glucose concentrations. World Journal of Microbiology & Biotechnology 3:257–260
50. Brühlmann F, Kim K et al (1994) Pectinolytic enzymes from actinomycetes for the degumming of ramie bast fibers. Applied & Environmental Microbiology 60(6):2107–2112
51. Kavuthodi B, Sebastian D (2018) Review on bacterial production of alkaline pectinase with special emphasis on *bacillus* species. Bioscience Biotechnology Research Communications 1: 18–30
52. Sakdeellaris G, Nikolaropoulos S, Evangelopoulos AE (1989) Purification and characterization of an extracellular polygalacturonase from lactobacillus plantarum strain BA 11. J Appl Bacteriol 1:77–85
53. Lonsane BK, Saucedo-Castaneda G et al (1992) Scale-up strategies for solid-state fermentation systems. Process Biochem 5:259–273
54. Liao C-H, Hung HY, Chatterjee AK (1988) An extracellular pectate lyase is the pathogenicity factor of the soft-rotting *bacterium Pseudomonas viridiflava*. Mol Plant-Microbe Interact 5: 199
55. Acuna-Arguelles M, Gutierrez-Rojas M et al (1995) Effect of water activity on exo-pectinase production by *aspergillus Niger* CH4 on solid-state fermentation. Biotechnol Lett 1:23–28
56. Nair SR, Rakshit SK, Panda T (1995) Effect of carbon sources on the synthesis of pectinase by aspergilli. Bioprocess Eng 13:37–40
57. Trejo-Hernfindez MR, Oriol E et al (1991) Production of pectinases by aspergillus Niger by solid fermentation on support. MicolNeotropApl 4:49–62
58. Debing J, Peijun L et al (2006) Pectinase production by solid fermentation from aspergillus Niger by a new prescription experiment. Ecotoxicol Environ Saf 2:244–250
59. Koboyashi T, Koike K et al (1999) Purification and properties of a low-molecular weight, high-alkaline pectate lyase from an alkaliphilic strain of bacillus. Bioscience Biotechnology Biochemistry 63:72–75
60. Friedrich J, Cimerman A, Steiner W (1990) Production of pectolytic enzymes by aspergillus Niger: effect of inoculum size and potassium hexacyanoferrate II-trihydrate. Appl Microbiol Biotechnol 4
61. Couri S, da Costa TS et al (2000) Hydrolytic enzyme production in solid-state fermentation by aspergillus Niger 3T5B8. Process Biochem 3:255–261
62. Silva D, Martins E da S, et al. (2002). Pectinase production by *penicillium viridicatum* RFC3 by solid-state fermentation using agricultural wastes and agro-industrial by-products. Braz J Microbiol 4
63. Kaur G, Kumar S, Satyanarayana T (2004) Production, characterization and application of a thermostable polygalacturonase of a thermophilic mould *Sporotrichum thermophile* Apinis. Bioresour Technol 94:239–243

64. Phutela U, Dhuna V et al (2005) Pectinase and polygalacturonase production by a thermophilic *aspergillus fumigatus* isolated from decomposting orange peels. Braz J Microbiol 36: 63–69
65. Patil SR, Dayanand A (2006) Production of pectinase from deseeded sunflower head by aspergillus Niger in submerged and solid-state conditions. Bioresour Technol 16:2054–2058
66. Palaniyappan M, Vijayagopal V et al (2009) Screening of natural substrates and optimization of operating variables on the production of pectinase by submerged fermentation using *aspergillus Niger* MTCC 281. Afr J Biotechnol 8:682–686
67. Banu AR (2010) Production and characterization of pectinase enzyme from penicillium chrysogenum. Indian J Sci Technol 4:377–381
68. Mandhania S, Jain V, Malhotra SP (2009) Culture optimization for enhanced production of microbial pectin Methylesterase under submerged conditions. Asian J Biochem 1:12–22
69. Martin N, Guez MAU et al (2010) Pectinase production by a Brazilian thermophilic fungus *Thermomucor indicae-seudaticae* N31 in solid-state and submerged fermentation. Microbiology 3:306–313
70. Patil NP, Chaudhari BL (2010) Production and purification of pectinase by soil isolate penicillium sp. and search for better agro residue for its SSF. Recent Researches in Science &Technology 2:36–34
71. Salariato D, Diorio LA et al (2010) Extraction and characterization of polygalacturonase of fomes sclerodermeus produced by solid-state fermentation. Revista Argentina de Microbiologia 42(1):57–62
72. Gomes J, Zeni J et al (2011) Evaluation of production and characterization of polygalacturonase by aspergillus Niger ATCC 9642. Food Bioprod Process 4:281–287
73. Demir H, Göğüş N, Tarı C, Heerd D, Lahore MF (2012) Optimization of the process parameters for the utilization of orange peel to produce polygalacturonase by solid-state fermentation from an aspergillus sojae mutant strain. Turk J Biol 36(4):394–404
74. Johsnon A, Odunfa S et al (2012) Production of cellulase and Pectinase from Orange peels by fungi. Nature and Science 10:5
75. Sharma N, Rathore M, Sharma M (2012) Microbial pectinase: sources, characterization and applications. Rev Environ Sci Biotechnol 1:45–60
76. Singh S, Mandal SK (2012) Optimization of processing parameters for production of pectinolytic enzymes from fermented pineapple residue of mixed *aspergillus* species. Jordan J Biol Sci 5(4):307–313
77. Vasanthi, Meenakshisundaram (2012) Optimization of pectinase enzyme production by using sour orange peel as substrate in solid-state fermentation. Asian Journal of Biochemical & Pharmaceutical Research 2:16–26
78. El-Batal AI, Osman EM &Shaima IAM1 (2013). Optimization and characterization of polygalacturonase enzyme produced by gamma irradiated penicillium citrinum. Journal of Chemical & Pharmaceutical Research 5:1: 336–347
79. Seifollah F, Khodaverdi G (2013) Pectinesterase production by aspergillus Niger: optimization of fermentation condition. Journal of Basic & AppliedScientificResearch 3(2):896–910
80. Siddiqui MA, Veena P, Arif M (2013) Polygalacturonase production from Rhizomucor pusillus isolated from fruit markets of Uttar Pradesh. Afr J Microbiol Res 7:252–259
81. Taskin M (2012) Co-production of tannase and pectinase by free and immobilized cells of the yeast Rhodotorula glutinis MP-10 isolated from tannin-rich persimmon (Diospyros kaki L.) fruits. Bioprocess Biosyst Eng 2:165–172
82. Demir H, Tari C (2014) Valorization of wheat bran for the production of polygalacturonase in SSF of aspergillus sojae. Ind Crop Prod:302–309
83. Ibrahim D, Weloosamy H, Sheh-Hong L (2014) Potential use of nylon scouring pad cubes attachment method for pectinase production by aspergillus Niger HFD5A-1. Process Biochem 4:660–667

84. Irshad M, Anwar Z et al (2014) Bio-processing of agro-industrial waste orange peel for induced production of pectinase by *Trichoderma viridi*; its purification and characterization. Turkish Journal of Biochemistry 1:9–18
85. Sudeep KC, Upadhyaya J et al (2020) Production, characterization, and industrial application of pectinase enzyme isolated from fungal strains. Fermentation 6(2)
86. Khatri BP, Bhattarai T et al (2015) Alkaline thermostable pectinase enzyme from aspergillus Niger stain MCAS2 isolated from Manaslu conservation area Gorkha. Nepal Springer Plus 4(1):488
87. Sethi BK, Nanda PK, Sahoo S (2016) Enhanced production of pectinase by aspergillus terreus NCFT 4269.10 using banana peels as substrate. 3 Biotech 6(1):36
88. El Enshasy HA, Elsayed EA et al (2018) Bioprocess optimization for pectinase production using aspergillus Niger in a submerged cultivation system. BMC Biotechnol 18(1):71
89. Margo P, Varvaro L, Avanzo C, Balestra GM et al (1994) Pectinolytic enzymes produced by *pseudomonas syringae pv*. Glycinea. FEMS Microbiol Lett. 117:1–6
90. Demir N, Nadaroglu H et al (2011) Purification and characterization of a pectin lyase produced by Geobacillus stearothermophilus Ah22 and its application in fruit juice production. Ann Microbiol 4:939–946
91. Ros JM, Schols HA et al (1996) Extraction, characterisation, and enzymatic degradation of lemon peel pectins. Carbohydr Res 2:271–284
92. Pandey A, Selvakumar P, Soccol CR, Singh Nee Nigam P (1999) Solid-state fermentation for the production of industrial enzymes. Curr Sci 77(1):149–162
93. Lonsane, Ghildyal (1992) Exoenzymes. In: Doell H, Mitchell D, Rolty C (eds) Solid-state cultivation. Elsevier. p. Chapter 11
94. Oriol E, Raimbault M et al (1988) Water and water activity in the solid-state fermentation of cassava starch by aspergillus Niger. Appl Microbiol Biotechnol 5–6:498–503
95. Roussos S, Olmos A et al (1991) Strategies for large scale inoculum development for solid-state fermentation system: conidiospores of Trichoderma harzianum. Biotechnol Tech 6:415–420
96. Gessesse A, Mamo G (1999) High-level xylanase production by an alkaliphilic bacillus sp. by using solid-state fermentation. Enzym Microb Technol 1–2:68–72
97. Pandey A, Nigam P et al (2000) Advances in microbial amylases. Biotechnol Appl Biochem 2: 135
98. Cavalitto SF, Areas JA et al (1996) Pectinase production profile of aspergillus foetidus in solid-state cultures at different acidities. Biotechnol Lett 3
99. Fonseca MJV, Said S (1994) The pectinase produced by Tubercularia vulgaris in submerged culture using pectin or orange-pulp pellets as inducer. Appl Microbiol Biotechnol 1:32–35
100. Ismail AMS (1996) Utilization of orange peels for the production of multienzyme complexes by some fungal strains. Process Biochem 7:645–650
101. Santier PH, Minjares A et al (1993) New approach for selecting pectinase producing mutants of aspergillus Niger well adapted to solid-state fermentation. Biotechnol Adv 3:429–440
102. Li T, Wang N et al (2007) Optimization of covalent immobilization of pectinase on sodium alginate support. Biotechnol Lett 9:1413–1416
103. Acuña-Argüelles ME, Gutiérrez-Rojas M et al (1995) Production and properties of three pectinolytic activities produced by aspergillus Niger in submerged and solid-state fermentation. Appl Microbiol Biotechnol 5:808–814
104. Pandey A (1992) Recent process developments in solid-state fermentation. Process Biochem 27:109–117
105. Acuna-Arguelles M, Gutierrez-Rojas M et al (1994) Effect of water activity on exo-pectinase production by *aspergillus Niger* CH4 on solid-state fermentation. Biotechnol Lett 1:23–28
106. Shi C, He J et al (2015) Solid-state fermentation of rapeseed cake with *aspergillus Niger* for degrading glucosinolates and upgrading nutritional value. Journal of Animal Science and Biotechnology 1

107. Heikinheimo R (1995) Characterization of a novel pectate lyase from Erwinia carotovora subsp. carotovora. Mol Plant-Microbe Interact 2:207
108. Pitkänen K, Heikinheimo et al (1992) Purification and characterization of Erwinia chrysanthemi B374 pectin methylesterase produced by Bacillus subtilis. Enzym Microb Technol 10:832–836
109. Hatada Y, Kobayashi T, Ito S (2001) Enzymatic properties of the highly thermophilic and alkaline pectate lyase pel-4B from alkaliphilic bacillus sp. strain P-4-N and the entire nucleotide and amino acid sequences. Extremophiles 2:127–133
110. Koboyashi T, Higaki N et al (2001) Purification and properties of a galacturonic acid releasing exopolygalacturonase from a strain of bacillus. BioscienceBiotechnolBiochem 65:842–847
111. Kuchenreuther JM, Stapleton JA, Swartz JR (2009) Tyrosine, cysteine, and S-adenosyl methionine stimulate in vitro [Fe-Fe] hydrogenase activation. PLoS One 4(10):e7565
112. Satyanarayana T., Vohra, & Ashima (2003). Phytases: microbial sources, production, purification, and potential biotechnological applications. Crit Rev Biotechnol 1: 29–60
113. Guo W, González-Candelas L, Kolattukudy PE (1995) Cloning of a new pectate lyase gene pelC from *fusarium solani f.* sp. pisi (Nectriahaematococca, mating type VI) and characterization of the gene product expressed in *Pichia pastoris*. Arch Biochem Biophys 2:352–360
114. Hasunuma T, Fukusaki EI, Kobayashi A (2003) Methanol production is enhanced by expression of an aspergillus Niger pectin methylesterase in tobacco cells. J Biotechnol 106:45–52
115. Margo P, Varvaro L et al (1994) Avanzo C, Balestra GM. Pectinolytic enzymes produced by pseudomonas syringae pv. Glycinea FEMS Microbiol Lett 117:1–6
116. Takao M, Nakaniwa T, Yoshikawa K (2000) Purification and characterization of thermostable pectate lyase with protopectinase activity from thermophilic bacillus sp. TS 47. BiosciBiotechnolBiochem. 64:2360–2367
117. Singh SA, Rao AGA (2002) A simple fractionation protocol for, and a comprehensive study of the molecular properties of two major endo polygalacturonases from aspergillus Niger. Biotechnol Appl Biochem 35:115–123
118. Favey S, Bourson C et al (1992) Purification of the acidic pectate lyase of Erwinia chrysanthemi 3937 and sequence analysis of the corresponding gene. J Gen Microbiol 138: 499–508
119. Nagai M, Katsuragi T et al (2000) Purification and characterization of an endo-polygalacturonase from *aspergillus awamori*. BiosciBiotechnolBiochem. 64:1729–1732
120. Koboyashi T, Koike K, Yoshimatsu T, Higaki N, Suzumatsu A, Ozawa T et al (1999) Purification and properties of a low-molecular weight, high-alkaline pectate lyase from an alkaliphilic strain of bacillus. BiosciBiotechnolBiochem 63:72–75
121. Singh SA, Plattner H, Diekmann H (1999) Exo polygalacturonate lyase from a thermophilic bacillus sp. Enzyme Microbial Technol. 25:420–425
122. Takao M, Nakaniwa T et al (2001) Molecular cloning, DNA sequence, and expression of the gene encoding for thermostable pectate lyase of thermophilic bacillus sp. TS 47. BiosciBiotechnolBiochem 65:322–329
123. Blanco P, Sieiro C, Villa TG (1999) Production of pectic enzymes in yeasts. FEMS Microbiol Lett 175:1–9
124. Pietro AD, Roncero MIG (1996) Purification and characterization of an exo-polygalacturonase from the tomato vascular wilt pathogen *fusarium oxysporum* f. sp. lycopersici. FEMS Microbiol Lett 145:295–298
125. Corredig M, Kerr W, Wicker L (2000) Separation of thermostable pectin-methylesterase from marsh grapefruit pulp. J Agric Food Chem 48:4918–4923
126. Barnby FM, Morpeth FF, Pyle DL (1990) Endopolygalacturonase production from Kluyveromyces marxianus. I. Resolution, purification and partial characterization of the enzyme. Enzyme Microbial Technol 12:891–897
127. Daniel IU, Chukwunonso AN et al (2014) Extraction, partial purification and characterization of pectinases isolated from aspergillus species cultured on mango (*Mangifera indica*) peels. Afr J Biotechnol 13:2445–2454

128. Alana A, Alkorta I et al (1990) Pectin lyase activity in a penicillium italicum strain. Appl Environ Microbiol 56:3755–3759
129. Dinnella C, Lanzarini G, Stagni A (1994) Immobilization of an endopectin lyase on g-alumina: study of factors influencing the biocatalytic matrix stability. J Chem Technol Biotechnol 59: 237–241
130. Sapunova LI, Mikhailova RV, Lobanok AG (1995) Properties of pectin lyase preparations from the genus penicillium. Appl Microbiol Biochem 31:435–438
131. Bruhlmann F (1995) Production and characterization of an extracellular pectate lyase from an Amycolata sp. Appl Environ Microbiol 61:3580–3585
132. Chen W-C, Hsieh H-J, Tseng T-C (1998) Purification and characterization of a pectin lyase from Pythium splendens infected cucumber fruits. Botanical Bull Academia Sinica 39:181–186
133. Moharib SA, El-Sayed ST, Jwanny EW (2000) Evaluation of enzymes produced from yeast. Nahrung 44:47–51
134. Dixit VS, Kumar AR et al (2004) Low molecular mass pectate lyase from fusarium moniliforme: similar modes of chemical and thermal denaturation. BiochemBiophys Res Commun 315:477–484
135. Dutta AC (1980) The tissue. Oxford University Press, London, pp 183–194
136. Bajpai P (1999) Application of enzymes in the pulp and paper industry. Biotechnol Prog 2: 147–157
137. Deshpande KS, Gurucharanam K (1985) Degumming of ramie fibers: role of cell wall degrading enzymes of aspergillus versicolor. Indian journal of botany Hyderabad 1:79–81
138. Baracat-Pereira MC, DantasVanetti MC et al (1993) Partial characterization of aspergillus fumigatus polygalacturonases for the degumming of natural fibers. J Ind Microbiol 3:139–142
139. Chesson A (1980) A review: maceration in relation to the post-harvest handling and processing of plant material. J Appl Bacteriol 1:1–45
140. Sharma HSS, Robinson E (1983) Fungal colonization during glyphosate induced desiccation and dew-retting of flax cultivars. Technical Report No. 2281:11
141. Ward OP, Fogarty WM (1974) Polygalacturonate lyase production by Bacillus subtilis and flavobacterium pectinovorum. Appl Microbiol 2:346–350
142. Chesson A (1978) The maceration of linen flax under anaerobic conditions. J Appl Bacteriol 2: 219–230
143. Rosemberg JA, De França FP (1967) Importance of galacturonic acid in controlling the retting of flax. Appl Microbiol 3:484–486
144. Ahmad M (1963) Studies on jute retting bacteria. J Appl Bacteriol 2:117–126
145. Jayasankar NP, Agate AD et al (1967) Microbial decomposition of pectic substances. V. Evidence for the role of micrococcus species in the retting of sisal and coconut husks. Journal of the Indian Institute of Science 49:10–17
146. Cao J, Zheng L, Chen S (1992) Screening of pectinase producer from alkalophilic bacteria and study on its potential application in degumming of ramie. Enzym Microb Technol 12:1013–1016
147. Tanabe H, Kobayashi Y et al (1986) Pretreatment of pectic wastewater from orange canning by soft-rot Erwinia carotovora. J Ferment Technol 3:265–268
148. Tanabe H, Yoshihara K et al (1987) Pretreatment of pectic wastewater from orange canning process by an alkalophilic bacillus sp. J Ferment Technol 2:243–246. -7
149. Horikoshi K (1990) Enzymes of Alkalophiles. In Microbial Enzymes and Biotechnology Springer, Netherlands, pp 275–294
150. Reid I, Ricard M (2000) Pectinase in papermaking: solving retention problems in mechanical pulps bleached with hydrogen peroxide. Enzym Microb Technol 2–4:115–123
151. Horn D, Linhart F (1996) Retention aids. In Paper Chemistry Springer, Netherlands, pp 64–82
152. Thornton J, Ekman R et al (1994) Polysaccharides dissolved from Norway spruce in thermomechanical pulping and peroxide bleaching. J Wood Chem Technol 2:159–175
153. Carr JG, Wood BJB (eds) (1997) Microbiology of fermented foods. Springer, US, pp 133–154

154. Godfrey A (1998) Production of industrial enzymes and some applications in fermented foods. In: Microbiology of fermented foods. Springer, US, pp 622–657
155. Paul NB, Bhattacharyya SK (1979) 47—the microbial degumming of raw ramie fiber. The journal of the textile Institute 12:512–517
156. Hellinger E (1954) Sporulating anaerobes on english flax. J Appl Bacteriol 1:6–13
157. Anuja V, Gupta R (2017) Pectinases and their biotechnological applications. In: Microbial enzyme Technology in Food Applications. CRC Press, pp 145–161
158. Renuka V et al (2009) Statistical optimization of substrate, carbon and nitrogen source by response surface methodology for pectinase production using aspergillus fumigatus MTCC 870 in submerged fermentation. Afr J Biotechnol 22:6355–6363
159. Teixeira MFS, Lima Filho JL, Durán N (2000) Carbon sources effect on pectinase production from aspergillus japonicus 586. Braz J Microbiol 4
160. Rombouts FM, Pilnik W, Rose AH (1980) Pectic enzymes. In: Economic microbiology, Microbial enzymes and bioconversions, vol 5. Academic Press, pp 227–282
161. Godfry T (1996) Introduction to enzymology, 2nd edn. Elsevier, pp 1–17

Applicability of Fungal Xylanases in Food Biotechnology

16

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Abstract

The purpose of food processing is to produce better-quality foods having good organoleptic properties. For centuries, enzymes have been in use for food processing. In the last three decades, xylan-hydrolyzing enzymes have been explored for their utility in food processing industries. Xylanases find applications in the bioconversion of lignocellulosics, fruit juice extraction and clarification, extraction of edible oils, saccharifying agro-residues, improving the quality of bread, and wine making. The use of xylanases in generating xylooligosaccharides (XOs) as prebiotics, which are known to modulate the gut microbiota, received significant attention in the recent years. The discovery of several microbial xylanases has enriched the CAZy database that accounts for more than 5000 different GH-10 and GH-11 xylanases from prokaryotic as well as eukaryotic microbes. This review focuses on the applications of microbial xylanases in food processing industries.

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Keywords

Xylan · Xylanase · Endoxylanase · Xylan hydrolysis · Xylooligosaccharides · Gut microbiome · Food processing · Baking · Fruit juice · Wine · Edible oils

16.1 Introduction

The Neolithic revolution, also known as agricultural revolution, started 12,000 years ago. During this age, humans started domesticating plants and livestock. They had domesticated barley, einkorn wheat, emmer wheat, flax, peas, and chickpeas under plant domestication. Neolithic period saw the domestication of sheep and cattle for meat, milk, and farming, but empirical studies predict the predated origin of bread [1]. Plants and animals were domesticated for human use. The aim of plant domestication was to meet the food and fiber demands for human consumption [2]. Ancient people processed the plant foods using heat, milling, fermentation, and others. These food-processing techniques altered the quality and nutrient availability of food [3]. Evolution in processing techniques of food led to the use of enzymes. Use of rennet in cheese making and diastase for starch hydrolysis are the two such prime examples of enzyme applications [4]. Enzymes are biological catalysts produced by living organisms. For centuries, enzymes have been used in food processing such as baking, brewing, wine making, curd making, cheesing making, tenderization of meat, clarification of fruit juices, and several others [5]. Enzymes play a complex role in food production. In food processing industry, enzymes help in improving the quality of food by enriching flavor, improving texture, and increasing shelf life in addition to shortening the process time. Application of biocatalysts in food industry is primarily meant for efficient utilization of raw substrate for making good-quality food products. It has been shown that the use of enzymes is beneficial from health point of view, and it also prevents the formation of acrylamide in French fries, coffee, and cereal breakfasts. In addition, the use of enzymes is considered as sustainable since they reduce the use of chemical additives. Amylase, lipase, protease, cellulase, xylanase, pectinase, catalase, asparaginase, esterase, and lactase are a few examples of commonly used enzymes in food industry. Earlier plant and/or animal extracts were used as sources of enzymatic preparations. The developments in microbiology and biotechnology changed this trend in favor of using microbial, native, and recombinant enzymes in food processing industries [6].

Plant cell wall is a complex structure made up of cellulose, hemicellulose, lignin, pectins, and proteins [7]. The composition of each cell wall component shows species to species variation in plants [7]. Hemicelluloses are made of diverse group of complex polysaccharides having heteropolymeric structure. Heteropolysaccharides can be classified according to the principal monosaccharides constituting the backbone such as xylan, xyloglucan, xylogalactans, and others [8]. Xylan is the most abundant hemicellulosic fraction in plant cell walls. The presence of a variety of linkages and side chains make xylan structurally complex. The presence of a variety of linkages affects the physiochemical properties of xylan

such as solubility and viscosity. Structural complexities limit the enzymatic breakdown of xylan. Complete hydrolysis of xylan requires synergistic actions of a set of enzymes [8]. Xylanases belong to glycoside hydrolases which catalyze the breakdown of glycosidic bonds of complex heteropolymeric xylan. Most of the industrially used enzymes are hydrolases.

16.2 Xylan Degrading Enzyme System

Structural complexity of xylan (Fig. 16.1) requires multifunctional xylanolytic system where glycosidic bond of xylan backbone is randomly catalyzed by endo-1,4-β-xylanase, while side chains are cleaved by accessory enzymes like acetylxylan esterase, α-L-arabinofuranosidase, and α-glucuronidase [9]. The carbohydrate-active enzyme (CAZy) database groups xylanases into glycoside hydrolase (GH) families [1, 10–20]. Xylooligosaccharide (XO)-degrading enzymes are mainly classified into GH families [1–3, 5, 21–27]. Amino acid sequence similarities are the basis for GH family classification (http://www.cazy.org). The mode of action of various enzymes in multienzymatic xylanolytic enzyme system includes the following: i) Endoxylanase (EC 3.2.1.8) is the most prominent enzyme that is involved in the reduction of degree of polymerization (DP). Reduction in the degree of polymerization varies in different xylan sources and highly dependent on the length and frequency of associated side chains [28]. The action of endoxylanases on xylan leads to liberation of lower XOs. The CAZy database categorizes endoxylanases mainly into GH-10 and GH-11 glycoside hydrolase families based on the hydrophobic cluster analysis of amino acids. Besides these, three more families (GH-5, GH-8, and GH-141) have also been shown to display β-1,4 endoxylanase activity. β-D-Xylosidase (EC 3.2.1.37) is another important class of xylanolytic enzymes that hydrolyzes XOs. Prolonged exposure of xylobiose and other XOs to β-D-xylosidase results in xylopentaose as an end product because of transglycosylation activity of β-xylosidase [29]. β-D-Xylosidases belong to high molecular weight (60–360 kDa) catalytic proteins and commonly found in a diverse group of microorganisms. Majority of the reported β-D-xylosidases are cell-bound which assist in direct transport of monomeric sugar xylose into the cytoplasm for its metabolism

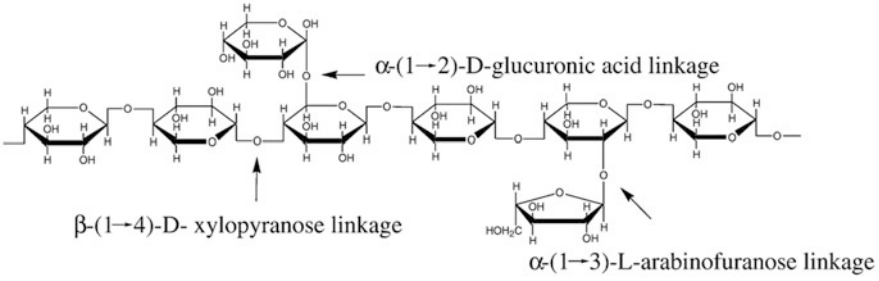

Fig. 16.1 Xylan structure with side chains

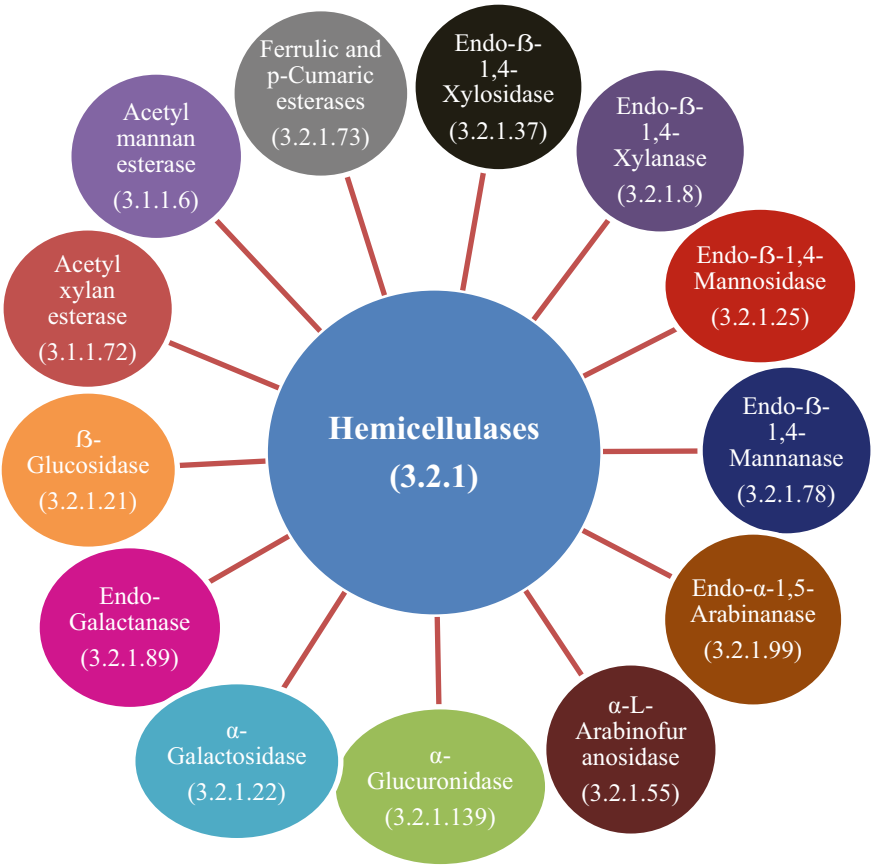

Fig. 16.2 Hemicellulase classification

[8]. However, extracellular β-xylosidases have also been reported from bacteria and fungi [30, 31]. β-D-Xylosidases exhibit three-dimensional structure of $(\beta/\alpha)_8$ type and have been placed into GH-1, GH-3, and GH-120 families of CAZy database (Fig. 16.2). β-Xylosidases play a significant role in generating D-xylose, which can be converted to xylitol. Xylitol has significant applications in pharmaceutical industry and extensively used in the treatment of dental caries [32]. Association of methylated and non-methylated glucuronic acid makes the xylan resistant as these side groups enhance the inaccessibility of xylan for endoxylanases and β-xylosidases [33, 34]. α-1,2-Glucuronidases overcome this restricted access by removing the associated side chains on xylan. In glucurono-xylan, α-linked glycosidic bonds anchor the glucuronic acid and/or methyl-glucuronic acid and xylosyl residues. α-Glucuronidase (EC 3.2.1.139) is among the side chain acting xylanases that cleaves α-linked glycosidic bonds of glucurono-xylan. GH-67 and GH-115 are the dedicated GH families for α-glucuronidases that fold into the 3-D structure of (β/

$\alpha)_8$ type [35]. Current updates of the CAZy database reveal the presence of 116 glucuronidases. Similarly, 578 glucuronidases belong to GH-67 family of CAZy. Arabinofuranosidase (AFase) is another accessory xylanolytic enzyme that removes α-linked L-arabinofuranose residues from the xylan backbone [21, 36]. Two forms of AFase exist in nature: exo-acting α-L arabinofuranosidase (EC 3.2.1.55) and endo-acting-1,5-α-L-arabinase (EC 3.2.1.99). The action of α-L arabinofuranosidases release arabinan as the end product, while 1,5-α-L-arabinase (EC 3.2.1.99) cleaves branched arabinans and p-nitrophenyl-α-L arabinofuranosides. According to CAZy database, there are six different GH families of AFase (GH-43, GH-62, GH-127, GH-137, GH-142, and GH-146). α-L-Arabinofuranosidase activity is exclusive to the GH-62 family, while β-L arabinofuranosidase activity is shown by the rest of GH family AFases [37, 38]. L-Arabinose finds applications in the food industry such as a dietary supplement, food ingredient, and flavoring agent. Synergetic action of AFases and endoxylanases, β-D-xylosidases, and amylases enhance liberation of XOs from softwood. Arabinose sugars are heavily used in food industries [39]. Acetyl xylan esterases (EC 3.1.1.72) assist in xylan hydrolysis by cleaving the acetyl group in the side chains. Similar to other side chains, acetylation and feruloylation of the xylan backbone also limit xylan hydrolysis. Acetylation and/or feruloylation hinder direct access of xylan main chain to endoxylanses and β-D-xylosidases [40]. An acetylxylan esterase (AXE) has been reported to hydrolyze the acetylated sugars [41]. However, AXEs do not show activity on acetylated pectin and mannan. Joseleau and colleagues [8] were the pioneers to report the microbial AXEs in 1985. The CAZy classifies acetylxylan esterases into CE-1 to CE-7 as well as in CE-12 of carbohydrate esterase (CE) family. Majority of the reported AXEs are of bacterial type [41, 42]. Very few AXEs have been studied for their applications in industries [43]. Zhang and his coworkers showed the utility of AXE in improving the solubilization of giant reed and wheat straw [43]. Complete hemicellulolytic system is depicted in Fig. 16.2.

16.3 Sources of Xylanases

Xylanase is widespread in nature ranging from prokaryotes to eukaryotes. Prokaryotic sources include archaea [44] and eubacteria [45], while eukaryotes include fungi [46], protozoa [47], algae [48], insects [22], snails [49], crustaceans [50], and plants [51]. Xylanase can be obtained from plants and animals, but microbes like bacteria and fungi produce xylanase in a high titer; therefore, microorganisms have always been the preferred sources of xylanolytic enzymes over others. There are added advantages of using microbes as a source of xylanolytic enzymes such as ease of handling, short doubling time, cheaper media, and very limited space requirement for growth [22].

Major sources for industrial enzymes are fungi and bacteria, where 50% are sourced from fungi and yeast, while 35% comes from bacteria. Contribution of animals and plants is 15% [52]. Commercially available microbial enzymes are sold

as enzyme preparations that contain desired enzymes along with some metabolites of producing strain, food grade preservatives, and stabilizers. There are a small group of bacterial and fungal strains which are used to produce most of the industrially relevant enzymes, since they are well characterized and easy to manipulate using conventional microbiological and genetic engineering approaches. These industrially relevant fungal groups include *Aspergillus*, *Trichoderma*, *Kluyveromyces*, and *Saccharomyces*, while primary bacterial groups are *Bacillus*, *Escherichia coli* K-12, and *Pseudomonas fluorescens which* are used for the industrial production of enzymes. Among microbes, bacteria have been extensively explored for producing xylanolytic enzymes. A plethora of microorganisms from bacteria to fungi produce a range of xylanases with varying temperature and pH stabilities. Xylanases have been successfully employed in the bioconversion of lignocellulosics, extraction of fruit juices and their clarification, extraction of edible oils, saccharifying agro-residues, improving the quality of bread, modulating gut microbiota, promoting the growth of beneficial gut commensals, and ripening of fruits. Xylanases find application in baking industry, where various xylanolytic enzymes along with other polysaccharide hydrolyzing enzymes can be used for ameliorating baking food items such as breads, cakes, crackers, pastries, pretzel, muffins, cookies, and other bakery products [53]. Recent trends include several options in food processing industry by incorporating whole wheat flour and other multigrain cereals (oat, mustard, millet, sorghum, etc.) into the raw dough to make healthier bread and cookies. For enhancing the nutritional value of such food products, a variety of xylanases can be added to the dough for hydrolyzing the bound sugars present in the cell walls of cereals. The food products produced would be of high nutritive value having natural forms of sugars along with fructo-/xylooligosaccharides.

Historically, strains used for industrial enzyme production are mainly derived by mutation and selection, but there are ongoing research efforts in developing microbial platforms using genetic engineering approaches. After the Asilomar Conference of 1975, recombinant DNA technology rapidly developed that helped in designing better bacterial and fungal strains which secrete high titers of industrial enzymes [54]. Developments in the field of next-generation sequencing (NGS) helped in achieving the design of efficient and high-throughput DNA libraries, while precise CRISPR/Cas-mediated DNA editing has been used to make genetic modification easy [23, 55].

16.4 Fungal Xylanases

A plethora of microbes have been reported to produce xylanases, including bacteria [24], actinobacteria [56], fungi [25], and yeasts [57]. Eukaryotic xylanases have been predominantly produced by filamentous fungi (*Thermomyces*, *Aspergillus*, *Fusarium*, *Myceliophthora*, *Penicillium*, *Trichoderma*) and yeasts [58–60]. Here it is important to mention that not all organisms produce the full set of xylan-degrading enzymes [61]. Filamentous fungi secrete industrially important xylanases that exhibit higher catalytic activity than those of yeast and bacteria. Additionally,

fungi produce a plethora of auxiliary enzymes simultaneously for the complete degradation of heteroxylans. Fungal genera which produce xylanases are *Thermomyces*, *Trichoderma*, *Aspergillus*, and *Fusarium*. The basidiomycete fungi can grow on diverse hemicellulosic substrates and produce extracellular hemicellulases. Degradation products of the hemicellulosic substrates are of immense interest for the food, pharmaceutical, and cosmetic industries. When *Phanerochaete chrysosporium*, *Trametes versicolor*, and *Ceriporiopsis subvermispora* are grown on plant cell wall materials or wood chips, they produce xylanolytic enzymes. Majority of xylanases are produced by fungi with low molecular weight within the range of 20–60 kDa [62]. While *Talaromyces emersonii* produces dimeric xylanases whose molecular masses range between 131 and 181 kDa [63]. The simultaneous production of cellulases and xylanases is a cause of concern for industry as it makes the process of xylanase purification expensive from these fungal sources. Xylanases from thermophilic fungi range from 21 to 47 kDa. Thermophilic xylanases show stability in a wide pH range (pH 4.0–9.0), but there is a predominance of xylanolytic activity at alkaline pH and high temperatures up to 60 °C. Recently characterized thermostable xylanase from *Malbranchea cinnamomea* exhibits stability at 70 °C [61].

16.5 Applications of Xylanases

The industrial use of xylanases for improving nutrient level of the fodder for animals was attempted during the 1980s and, later, in biofuels, food, and other industries. Extracellular xylanases attack lignocellulosics and enhance the quality of feed and food by enriching them with metabolizable sugars, amino acids, Ca^{2+}, phosphorous, and other nutrients. Biophysical properties of xylanases are crucial and decide their suitability for applications in various industries. With the increased demand for xylanases, they are being commercialized in single as well as in combinations with other polysaccharide-hydrolyzing enzymes.

16.6 Xylanases in the Baking Industry

Bread and bakery products are the most popular food items consumed all over the world. Traditionally, bread is manufactured using refined flour. Refined flour contains very low level of dietary fibers, minerals, and essential nutrients. Bread manufacturers are looking at high-fiber and more nutritious bread. But whole wheat bread also contains human-indigestible polysaccharides (non-starch polysaccharides) which are a major problem for manufacturers as these reduce the loaf volume and increase the firmness of crumb and crumb appears darkened that reduces the customers' appeal [64], although demand for high-fiber, low-calorie sugar-free bread is on the rise. Cellulose, insoluble heterogeneous arabinoxylan, lignin and β-D-glucans are major constituents of whole wheat grain. Non-starch polysaccharides are dietary fibers present in cereals used in bread making.

Non-starch polysaccharides are mainly made up of arabinoxylans, β-glucan, and arabinogalactan-peptide complex. Arabinoxylans constitute the major portion of non-starch polysaccharide present in cell walls of cereal grains such as wheat and rye [65, 66]. Based on solubility, arabinoxylans can be classified as water-non-extractable arabinoxylan (WN-AX) and water-extractable arabinoxylan (WE-AX). Most of the wheat arabioxylans are WN-AX. WN-AX constitutes 70–80% of the wheat arabinoxylan, while 20–30% is WE-AX. The β-1,4 linked D-xylopyranosyl is the backbone of arabinoxylans substituted with α-L-arabinofuranose residues at the C(O)3 and/or C(O)2 positions [67–69]. The proportion of arabinoxylan in bread made from wheat flour is only about 1.5–2.5% [65], but the effect of arabinoxylan on bread making is significant, since it affects the rheological properties of dough. It has been shown that viscosity enhancing, gelling properties, and water holding capacity of arabinoxylan improve the bread quality by increasing the loaf volume and firmness of crumb [70]. The texture of bread is also affected by arabinoxylan which is mainly because of gas retention properties of the dough. For baking industry, WN-AX negatively influences the bread characteristics, while WE-AX impacts positively [67]. Therefore, xylanase is often used to convert WE-AX into solubilized AX (SAX). The application of xylanase positively impacts AX structure and functionality and, thus, results in increased loaf volume [71]. The concentration of arabinoxylan in bread, molecular mass, and type of bread flour determines the impact of arabinoxylan [72]. Hence, the polysaccharide arabinoxylan determines the bread quality and properties of dough. For baking industry, arabinoxylans and arabinogalactans are critically important as these enhance water holding capacity of dough that ultimately improves the dough stability. Further, arabinoxylans help in reducing starch retrogradation, increases the loaf volume, and enhances the shelf life and storage stability of bread [73].

To solve the negative effect of WN-AX, endoxylanases are used. Application of xylanases improves the dough quality by altering the elasticity of gluten protein network by acting on WN-AX. In baked products, gluten is responsible for texture, shape, and chewiness. Gluten protein network affect the stability, extensibility, flexibility, and cohesiveness of dough. During dough formation, short gluten flours negatively affect the bread quality as dough made from short chain dough proteins are fragile, and dough is not much stable when compared with dough formed using long chain gluten protein flour. Xylanase supplementation in the presence of reducing agent (L-cysteine) enhanced the elasticity, porosity, and bread volume [74]. Endoxylanase functionality in bread making is mainly affected by substrate specificity, enzyme dosage, and selectivity toward enzyme inhibitors [75]. GH10 xylanases are known for broader substrate specificity, while GH11 xylanases are more specific in nature [76]. Another factor that affects the endoxylanase activity in breadmaking is the presence of endoxylanase inhibitors. Endoxylanases behave variably toward the proteinaceous inhibitors [77, 78]. *Triticum aestivum* endoxylanase inhibitor (TAXI) and endoxylanase inhibiting protein (XIP) show different inhibition specificities toward microbial endoxylanases. XIP is more selective toward fungal GH 10 and GH 11 enzymes, while TAXI inhibits GH11 family of xylanases. In addition to these inhibitors, there are other inhibitory proteins that

affect the functionality of endoxylanases in bread making [79, 80]. Activity profile and functionality of xylanase are affected by inhibition sensitivity of xylanase toward proteinaceous inhibitors [75].

Xylanases are used in combination with amylases, lipases, and many other oxidoreductases. Application of xylanases in combination with other enzymes brings out the desirable rheological and organoleptic properties [20]. The complete hydrolysis of arabinoxylans requires synergistic action of hemicellulases. Endo-1,4-β-xylanases of GH-11 cleave arabinoxylan backbone randomly resulting in long chain arabinoxylooligosaccharides, while GH10 xylanases act on short-chain arabinoxylooligosaccharides. These arabinoxylooligosaccharides are converted into xylose monomers by β-D-xylosidases (EC 3.2.1.37) from the non-reducing end, while arabinose residues are removed by the action of α-L-arabinofuranosidases (EC 3.2.1.55). Moreover, ester linkages between arabinose and ferulic acid can be efficiently removed by ferulic acid esterases (EC 3.1.1.73) [76, 81]. For baking industry, xylanases with affinity toward WN-AX are preferred over WE-AX, as the former remove the insoluble arabinoxylans [67, 71]. Additional benefit of xylanase application is increasing the concentration of arabinoxylooligosaccharides in bread. Arabinoxylooligosaccharides beneficially impact human gut microbiota as prebiotics.

Baking industry is looking for promising approach of synergistic enzyme cocktail to improve baked products. These enzyme cocktails must improve the rheological properties and nutritional value of baked products [82]. Our understanding of the dough formation and baking mechanisms has increased at the molecular level and also the interactions between different components of dough and enzymes used, which demands further improvisation leading to efficient and economically tailored enzymes alone or in combinations for baking industry. In general, cocktails of amylases, xylanases, and lipases have been used in bread making [83, 84].

Arabinoxylan found in wheat flour endosperm is water soluble, while water-insoluble arabinoxylan fiber can be found in cell wall polysaccharides. Enzymatic interactions of xylanases with insoluble arabinoxylan make it soluble. Arabinose, generated after the enzymatic activity, interacts with water in the dough and increases the volume. The increase in the volume of dough aids in creating more uniform crumbs and decrease in the dough firmness. The bacterial xylanases (GH-10 and GH-11) share the major chunk over the other sources of xylanases. GH-10 xylanases fold into TIM-barrel structure, while those of GH-11 are of β-jelly roll structure.

Staling is a major area of concern for baking industry. When bakery products are stored for longer duration, the baked products start showing the crust and crumb state resulting in the spoilage of baked products. In staling, freshness, aroma, moisture, and elasticity are lost. Studies show that the application of xylanase softens the dough; the softening results from the action of arabinoxylan breakdown [85]. It has been shown that xylanase treatment helps in improving the organoleptic properties of bread by reducing the staling rate and also increasing the shelf life of baked products [86]. Fungal xylanase in association with α-amylase or alone showed an increase in crumb softness and larger loaf volume of the baked bread [87]. When

barley bread was compared with whole grain wheat bread, it was found that the application of cocktail of enzymes enhances the total and soluble fiber content in case of barley bread. Further, enzyme cocktail treatment assisted in increasing the volume and crumb softness of whole grain wheat bread and barley bread [88]. Thermostable fungal xylanase was used along with various metal ion and amino acid concentrations that led to enhanced moisture content (32.33%) and improvement in nutritional value of bread [16]. Increase in dough volume, enhancement in moisture content, and reduction in crumb firmness were also observed when a xylanase from *Aureobasidium pullulans* was used; additional but minor decline was also observed in elasticity and gumminess [89]. When the dough was treated with *Aspergillus niger* xylanase, improvement was seen in elasticity, moisture content, stiffness, and coherency of dough [90]. In another study, where pentosanases and phospholipases were used in combination, improvement in elasticity, moisture content, stiffness, and coherency of bread was recorded [11]. Xylanase, when blended with other carbohydrate-active enzymes like cellulase and amylase, allowed better dough processing than the individual xylanase treatment. When xylanase was used along with amylase and cellulase, there was 42% increase in wheat flour dough extensibility [86]. The specific bread volume increased by 25.6% and crumb hardness was decreased by 46% when GH11 xylanase of *Chaetomium* sp. CQ31 was expressed in *Aspergillus niger* and used in bread making [87]. In a site-directed mutagenesis (SDM) study, it has been shown that the nature of the xylanase binding site significantly affects the catalytic efficiency of xylanase. Using SDM approach, modification was introduced in secondary binding site of the xylanase. This modification in secondary binding site increased relative activity toward WN-AX as compared to the wild-type xylanase without affecting other biochemical properties. Mutation in the secondary binding site caused an enhancement in substrate selectivity. The mutant enzymes increased loaf volume in low dosage as compared to the wild-type xylanase [91].

Papad is a part of traditional Indian food. It is thin, crispy having wafer-like texture [14] that showed the utility of xylanase in papad manufacturing. Papad manufactured from the black gram is arabinoxylan rich. The presence of a high amount of arabinoxylan in black gram is responsible for very hard dough formation in manufacturing papad [14]. The addition of xylanase to black gram flour improved the dough properties significantly. Xylanase helped in reducing the hardness of papad dough and also water requirement. Further, xylanase addition to dough reduced the oil consumption while frying the papad. There was no change in taste, color, or the texture of papad, when the dough was supplemented with xylanase.

16.7 Xylanases in Juice Industry

Fruits and vegetables are vital part of a healthy diet. In a healthy diet, fruits and vegetables are sources of nutrients and bioactive compounds such as phytochemicals, minerals, vitamins, and fibers. Fruits and vegetables are nutrient rich but perishable, which forced the food technologists to process them into various

processed food products. Juices are the most commonly produced form of fruits and vegetables which can be consumed over a longer period of time. In the mid-1930s, when the juice industry started delivering juice to the market, it faced a challenge of low juice yield and filtration for acceptable quality of juice [9].

Sugary and carbonated drinks possess adverse health impact, and also, they are less on nutrition as compared to vegetable and fruit juices. It has been seen that fruit and vegetable juices are gaining high popularity over addictive and aerated drinks especially in developed countries [92]. There is, however, no conclusive evidence that proves the associated health benefits of fruit and vegetable juices [93–95], although juices are perceived as healthier option over sugar sweetened beverages. In fruits and vegetables, there are cellulose, hemicelluloses, pectin, proteins, lipids, and other biomolecules which contribute to the cloudiness of naturally extracted fruit juices. These products of fruits and vegetables contribute significantly to the color, aroma, and the flavor of juices. Turbidity or cloudiness and haze formation serve as their quality indicators. Turbidity is due to plant cell wall pectin polysaccharides and other polysaccharides present in juices after mechanical extraction [96], while protein-polyphenol interactions result in haze formation [97]. Plant cell wall-degrading enzymes such as pectinase, cellulase, and hemicellulases are employed in order to remove the undesired characteristics of juices (cloudiness, turbidity, and haze). The application of these enzymes contributes to the quality of juices in terms of juice clarity and yield [98, 99]. It has been shown that xylanases can be efficiently employed for clarification and extraction of fruit juices [98, 100, 101]. The addition of xylanases to the pulp from various sources leads to improvement in yield, sugar content, acidity, clarity, and filterability of the juices along with a significant reduction in viscosity. In a recent report, various carbohydrases (β-mannanase, endo-xylanase, α-galactosidase, β-xylosidase, and β-glucosidase) of *Aspergillus quadrilineatus* RSNK-1 have been employed to enhance the reducing sugar content and enrichment of various common fruit pulps [99]. Incubation time for the treatment of juice with xylanase varies according to the type of fruit pulp processed. One group observed that the maximum incubation time of 150, 120, and 90 min for orange, mosambi, and pineapple juice, respectively [102]. Prolonging the optimized incubation time leads to the formation of complexes of proteins with carbohydrate as well as other proteins [100]. Majority of investigations showed that 30–90 min of xylanase treatment yields favorable results [101]. It should be mentioned here that fruit industry prefers acidic and thermostable xylanases for downstream processing of fruits. Several fungal and bacterial xylanases have been reported to be acidic and thermostable, therefore, suitable for their application in fruit industries [99, 103, 104]. Immobilized xylanase obtained from *Trichoderma longibrachiatum* was used to clarify orange juice at higher temperature indicating that immobilization of enzyme improves the thermostability [10]. There are also reports on the utility of neutral and alkaline xylanases in clarification of juices from various fruit pulps [98, 101].

16.8 Xylanases in Wine Industry

Wine is an alcoholic drink made from fermentation of grape juice. There are also wines made from other fermented fruit juices. In wine production, enzymes are mainly used for mashing and juice and wine treatment. The applications of enzymes not only influence the extraction, clarification, and filtration but also the stabilizing processes and aroma release resulting in quality wines. Most widely used enzymes for hydrolyzing the polysaccharide cell walls are pectinases, β-glucanases, and hemicellulases. Pectin, glucan, and xylan are responsible for viscosity, turbidity, and filter stoppages. Pectinase was the first endogenous enzyme used in wine making during the 1940s, while PECTINOL 59 L was commercialized by Rohm and Haas with a focus on wine industry. Endogenous enzymes were not so effective, thus resulted in poor clarification and methanol formation [105].

The first commercial use of enzymes in wine production started in the late 1960s after which many enzymes commercialized to cater to the needs of the wine industry. The first commercial enzyme mixture contained pectin esterase, polygalacturonase (PGase), pectin lyase (PL), and hemicellulase [13]. During initial phase of enzyme application in wine industry, the use of enzymes was limited to mash treatment, but soon enzymes have been used in making low alcohol wines, protein stabilization, color stabilization, flavor enhancement, aroma release, and others [18].

Wine making begins with crushing of grapes as grapes are the majorly used raw material. Cell wall-degrading enzymes cannot degrade grapes if they are not crushed. In wine making point of view, grape skins are very important as they hold flavanoids, aromas, aroma precursors, and tannins [106]. Grape berry comprises pectocellulosic cell walls susceptible to enzymatic degradation. These pectocellulosic cell walls are structurally complex in which cellulose microfibrils are anchored by a matrix of hemicelluloses (xylan, xyloglucan, mannan, etc.) and pectin. Neutral sugars like arabinose and galactose are also part of this complex pectocellulosic structure which hamper extraction, clarification, and filtration of the juice at the time of wine making. Vacuoles present in pectocelluloses are the source of sugars and organic acids. Hemicellulose and cellulose are present in varying amounts in different grape cultivars.

There are many commercial enzyme cocktails available for use in wine making industry. Middle lamellar pectin is disintegrated by protopectinases which results in softening of tissue [107]. Further, pectinases in association with cellulases and hemicellulases (xylanase, xyloglucanase, and galactanase) efficiently disintegrate the primary cell wall of grapes [17]. Hemicellulase and cellulase in pectinase mixture are desired in red grapes for extracting maximum skin cell contents, but are unwanted in white grape maceration to limit overextraction. In one study, Cytolase 219 (cocktail of xylanase, cellulase, and pectinase) was used for macerating three varieties of white grapes (Soave, Chardonnay, and Sauvignon). Application of Cytolase 219 improved extraction of juice from 10 to 35% and filtration rate from 70 to 80%. There was decrease in viscosity from 30% to 70% and pressing time reduced to 50 min from 120 min [13]. This application of Cytolase 219 also resulted in energy saving as energy demand comes down to 20% from that of 40% during

cooling of fermenter, and the end product (wine) was stable in nature. This report has shown that if right amount of cell wall-degrading enzymatic preparations (hemicellulase, cellulase, and pectinase) is used, the total juice yield, grape pressability, and settling rate can be significantly improved. Aroma of wine is another sensory interest which characterizes the quality of wine. Important aromatic compounds of wine include monoterpenes, sesquiterpenes, norisoprenoids, methoxypyrazines, phenylpropanoids, higher alcohols, and esters [108, 109]. These aroma molecules are present as odorless glycoconjugates in grapes. Monoterpenes are the aroma molecules in white wine, and to enhance the aroma of white wine, immobilized β - D –glucosidase and α - L – arabinofuranosidase were used for degrading glycoconjugates. In enzymatic hydrolysis of glycoconjugates, first the monoterpenyl β - D - glucoside moieties were released by the action of α - L –arabinofuranosidase and in subsequent event, monoterpenyl β - D –glucosidase releases the monoterpene aroma molecule [110].

16.9 Xylanases in Edible Oil Extraction

Oil extraction at industrial level from plant sources requires recovery of oil from oil bearing parts of plants, refining, modification, and stabilization that results in refined, bleached, and deodorized oil devoid of free fatty acids, pigments, phosphatides, etc. Mechanical pressing and solvent extraction are two conventional methods of making edible oils. Industry prefers solvent extraction for oil recovery which uses n-hexane with over 96% of crude oil yield. But high level of n-hexane exposure affects the human central nervous system, and excessive use of this solvent is also not environment friendly as it emits volatile organic compounds into the environment [111]. Therefore, oil industry is looking for alternative eco-friendly green, safe, and health compliance technology for efficient oil recovery. Aqueous enzymatic extraction (AEE) is one such alternative source of oil extraction technology gaining impetus in oil industry.

In standard aqueous oil extraction, water is used as extraction solvent to remove proteins and other oilseed components which are soluble in water and leaving behind an emulsion or free oil for separation. Lipid molecules are amphiphilic in nature, i.e., they have polar hydrophilic end and non-polar hydrophobic ends that aids in forming oil emulsion. This oil emulsion is later de-emulsified to recover oil from emulsion. Oil recovery using aqueous oil extraction is in the range of 26%–66% which is very low when compared with solvent (n-hexane) extraction method. Aqueous enzymatic extraction is promising oil separation technology. The low yield of oil is mainly attributed to water's inability to completely degrade the cell wall components of oil-bearing part. Enzymes are used to overcome this limitation of water that helps in efficient degradation of oleaginous material. In aqueous enzyme extraction method, the best suited enzyme combinations are used for maximum oil recovery, and for that, it is important to know the biochemical composition and percentage of primary and secondary cell wall as well as oil bearing organelles/components [112]. Cell wall components are mainly made up of cellulose, hemicellulose, and pectin. To degrade

cell wall components, cellulases, hemicellulases, and pectinases are required for AEE, but different oleaginous plants have different cell wall biochemical composition of cellulose, hemicellulose, and pectin. The enzyme-assisted pressing employs a mixture of cellulases and hemicellulases. Representative oil crop feedstocks are presented in Table 16.1 and enzyme mixture containing xylanases in Table 16.2, where enhanced oil recovery has been recorded by AEE.

16.10 Xylan-Derived Xylooligosaccharides

The random hydrolysis of heteroxylan by endoxylanases generates lower xylooligosaccharides (XOs), which exhibit a huge potential as prebiotics that can be included as a food supplement in a variety of food products. XOs constitute 2–7 xylose units linked through β- [90, 113]-linkages. XOs are the only type of nutraceutical that can be produced by hydrolyzing agro-industrial lignocellulosic biomass [12]. The global market for such XOs was valued at USD 88.1 million in 2016, which is expected to grow at 5.3% CAGR and may reach USD 120 million by the end of 2022. There are several reports on xylooligosaccharide production from lignocellulosic biomass by using enzymatic hydrolysis. Agro-residues such as wheat bran, rice husk, corn cobs, wheat straw, sugarcane bagasse, and cotton stalks grabbed attention as rich sources of various XOs in a cost-effective manner [114]. Microbial xylanases have always been a choice over chemical and autohydrolysis methods for producing XOs because methods other than microbial enzymes contaminate the XOs by adding monomeric sugars and hydroxymethyl furfurals (HMFs) [114–116]. Enzymatic mode of XO production has been reported from various bacteria and fungi, where acidic to alkaline xylanases of varying optimum temperatures for activity have their potential in generating XOs from agro-residues. In our laboratory, several thermostable and alkaline xylanases of *Geobacillus thermoleovorans* [117], *Geobacillus thermodenitrificans* TSAA1 [118], and *Bacillus halodurans* TSEV1 [119] have been employed for releasing XOs from various agro-residues. In an interesting investigation [120], XOs have been produced from enzymatic hydrolysis of corncobs promoting the growth of four gut commensals (*Bifidobacterium adolescentis*, *B. bifidum*, *Lactobacillus fermentum*, and *L. acidophilus*), therefore supporting their role as prebiotics. Reddy and Krishnan [121] reported the production of XOs from wheat bran and groundnut oil cake. XOs of corn cobs have been detected for anti-oxidant properties by having concentration-dependent-free radical scavenging activity [19]. Immobilized enzymes/cells allow repetitive use along with functional efficiency in a cost-effective manner [122]. Microbial xylanases have also been immobilized for XO production from various natural materials [114, 123].

The fungal system has also been used extensively as sources of xylanolytic enzymes for generating lower oligosaccharides from xylan. *Aspergillus*, *Trichoderma*, and *Penicillium* represent dominant genera for secreting acidic xylanases and have been investigated for XOs production [123–125]. Synergistic effect of recombinant xylanase (XynC) of *P. funiculosum* and arabinofuranosidase

Table 16.1 Commercial xylanases and their applications

S. no.	Commercial name	Family	Specific activity	Company	Microorganism	Applications
1.	E-XYLATM	GH-10	–	Megazyme, USA	*Thermotoga maritima*	Food industry and feed industry
2.	E-XYTR1	GH-11	~ 230 U/mg 40 °C, pH 4.5 on wheat arabinoxylan		*Trichoderma viride*	Food and feed, biofuel and pulp industry
3.	E-XYTR3	GH-11	> 100 U/mg 40 °C, pH 6.0 on wheat arabinoxylan		*T. longibrachiatum*	
4.	E-XYLAA	GH-11	~ 30 U/mg 40 °C, pH 4.5 on wheat arabinoxylan		*Aspergillus aculeatus*	
5.	E-XYAN4	GH-11	~ 80 U/mg (40 °C, pH 4.5 on wheat arabinoxylan		*Aspergillus niger*	
6.	E-XYRU6	GH-11	~ 380 U/mg (40 °C, pH 6.0 on wheat arabinoxylan		Rumen microorganisms	
7.	E-XYNBCM	GH-11	1500 U/mg 50 °C, pH 6.0 on wheat arabinoxylan		*Neocallimastix patriciarum*	
8.	BIOXYLANASE BKF, BIOXYLANSE BAC	–	–	Biocon, India	*Trichoderma longibrachiatum*	Bread and biscuits
9.	BIOPECTINASE FR	–	–	Biocon, India		Fruit processing
10.	POWERbake@8000	–	–	DuPont Danisco, Denmark	Fungal, bacterial	Bakery
11.	Multifect XL	–	–	Genecor International Europe Ltd, Finland	*Trichoderma longibrachiatum*	Food industry
12.	Xylanase250	–	–	Hankyu Bioindustry Co. Ltd. Japan	*Trichoderma viride*	Baking industry and macerating vegetables and fruits
13.	Bioxylanase	–	–	Quest International, Ireland	*Trichoderma reesei*	Brewing and animal feed industry
14.	Solvay pentosanasa	–	–		*Trichoderma reesei*	Starch and baking industry

(continued)

Table 16.1 (continued)

S. no.	Commercial name	Family	Specific activity	Company	Microorganism	Applications
				Solvay Enzymes GmbH & co., Germany		
15.	Irgazyme 40/4X	–	–	Genencor, USA	*A. niger* and *Trichoderma viride*	Food industry
16.	Nutrilife	–	–	BASF, USA	–	Food industry

Table 16.2 Enzyme mixtures containing xylanase for AEE of oil

S. no.	Brand name	Manufacturer	Presence of xylanase
1.	Nutrase	Nutrex	Xylanase
2.	Kemzyme	Kemin	Xylanase is part of mixture
3.	Maxoliva	Centerchem	Xylanase is part of mixture
4.	Multifect	ChiralVision	Xylanase is part of mixture
5.	Natuzyme	Bioproton	Xylanase is part of mixture
6.	Protizyme	Not available	Xylanase is part of mixture
7.	Rapidase LIQ Plus	Not available	Xylanase is part of mixture
8.	Viscozyme L	Novozymes	Xylanase is part of mixture
9.	Flavourzyme 1000 L	Novozymes	Xylanase is part of mixture
10.	GC440	DuPont	Xylanase is part of mixture

(AbfB) of *A. niger* showed to release a significant high level of XOs from pretreated sugarcane bagasse [124]. Similarly, GH10 endoxylanase of *Thermoascus aurantiacus* heterologously expressed in *Pichia pastoris* was used to produce XOs using pretreated sugarcane bagasse [126]. Commercial xylanase of *Trichoderma viride* produced 1.15 mg/ml of XOs from alkali-treated sugarcane bagasse at 40 °C at acidic pH 4.0 in 8 hrs [127]. While xylanase obtained from *Aspergillus flavus* AW1 was used to produce XOs with antioxidant activity [128]. A triploid plant *Populus tomentosa* has been reported to be a source of XOs by using xylanase of yeast *Pichia stipitis* [129]. A commercial fungal xylanase of *T. viride* showed its potential by releasing 1.7 mg/ml of xylobiose and 1.1 mg/ml of xylotriose from a grass *Sehima nervosum* [127].

Besides endoxylanases, β-xylosidases and glycosynthases have also been reported for synthesis of XOs of varied properties [26, 27, 130, 131]. Two β-xylosidases of *Aspergillus nidulans* FGSC A4 produced five novel transxylosylation products using pNPX as electron donor and various mono-/disaccharides and sugar alcohol as acceptors [26]. Similarly, a high molecular weight (250 kDa) β-xylosidase of *Aspergillus* spp. displayed transxylosylation activity by synthesizing (PNP) β-1,4-D-xylooligosaccharides with a degree of polymerization of 2–7 [45]. Synergistic action of two β-xylosidases (Xyl43A and Xyl43B) and one xylanase (Xyn11A) of *Humicola insolens* results in 1.29-fold increase in reducing sugars from various xylan sources [132]. Recently β-xylosidase of *Humicola grisea* var. *thermoidea* was successfully employed for saccharification of sugarcane bagasse for generating fermentable sugars [133].

Kim et al. were the pioneers in reporting glycosynthase-mediated XO production [27]. Glycosynthases exhibit an irreversible mode of catalysis, where a xylobiosyl fluoride donor transfers the xylobiosyl moiety either to *p*-nitrophenyl or β benzylthio-xylobioside to produce XOs of varied length [114]. One group mutated the *Talaromyces amestolkiae* xylanase by replacing glutamic acid with glycine at 236th position that led to generation of glycosynthase. This mutated version of xylanase was further used to produce antioxidant XOs [134]. A handful of reports are available on β-xylosidase and glycosynthase-mediated XO production, thus

encouraging researchers to search for more such enzymes. Xylooligosaccharides are in massive demand due to their recognition as prebiotic for benefiting human health [114]. Current food industries are exploring the properties of XOs by incorporating them into various solid and liquid-based diets especially in dairy and baking industries. Incorporation of XOs into yogurt significantly affects various physiochemical properties such as pH, total solids, moisture content, and overall taste. Various aspects of prebiotics including XOs have been extensively reviewed [113] for their emerging role as prebiotics. Wheat-based cookies were prepared using varying concentrations of XOs that resulted in enhancement of crude and dietary fiber contents in cookies by 14 and 35%, respectively [15]. In addition, moisture content and shelf life of the cookies have also been ameliorated. XOs are less sweet, acidic, and thermostable in nature and require a very low amount as an additive to foods; therefore they can act as a sugar replacer in various food materials and drinks [135]. The antimicrobial activity of XOs further signifies the importance of these oligos in increasing the shelf life and stability of processed foods [136].

Xylitol is another important compound produced from xylose fermentation, which has multiple applications in food, odontological, and pharmaceutical industries. Xylose can be biotransformed into xylitol by using various yeast strains in a cost-effective manner [137], [138]. Xylitol is used as a sugar substitute for diabetics due to having only one-third calorific value as that of sucrose [138]. It has other distinctive pharmacological characteristics by preventing tooth decay and ear infection. Approximately two-thirds of ingested xylitol can be utilized by gut microorganisms which show prebiotic effects by reducing the level of blood glucose, cholesterol, and triglycerides. In addition, the production of several short chain fatty acids (SCFA) such as acetate, propionate, and butyrate acts as energy sources and lowers the pH of the colon and thus reduce the bioavailability of several cytotoxic compounds of alkaline nature [138].

16.11 Safety and Regulation of Food Enzymes

A commercial enzyme preparation for food industry requires safety and regulatory evaluations meeting international standards [139]. Most of the currently available industrial enzymes are microbial in origin, and microbial hosts are used to manufacture these enzymes. Industrial production of food enzymes requires large-scale fermentation to obtain high titer of enzymes. Enzymes obtained after fermentation are concentrated and purified using purification technology, but some other microbial metabolites of producing strain generated during fermentation process also remain with it. Further, preservatives and stabilizers are also added to enzyme mixtures. In this light, it is important to evaluate safety of enzyme preparations [140]. While evaluating the safety of the food enzymes, evaluator focuses on the enzyme-producing organism, desired enzyme, and its properties and manufacturing process. Enzyme-producing strain must be of generally recognized as safe (GRAS) [141], or it is one of the GRAS organism used for enzyme production in food applications, and manufacturing process must follow the good industrial

large-scale practice (GILSP). In case of recombinant strains, regulatory agencies desire a marker-free, minimal foreign DNA containing stable genome of host organism that produces the desired enzyme [142]. Further, allergenicity and toxicity of enzymes must be evaluated for safety considerations before use in food applications [140, 141].

16.12 Commercialized Xylanases

The market of carbohydrate hydrolyzing enzymes has been increasing rapidly that would share half of the total market of enzymes in the near future. Global market of food enzymes is expected to reach USD 3.6 billion by 2024 (Global Market Insights, Inc.). At present, the xylanase market is projected to grow at a compound annual growth rate (CAGR) of 6.6%. Several companies commercialize xylanase, where major share is that of Alltech Inc., USA (https://www.alltech.com/); Adisseo, France (https://www.adisseo.com/en/); BASF Enzymes LLC, California, USA (https://www.basf.com/global/en/products/segments/nutrition_and_care/ nutrition_and_health/enzymes.html); Novozymes, Denmark (https://www.novozymes.com/en); Enzyme Development Corporation, New York, USA (http://www.enzymedevelopment.com/); Genencor, New York, USA (http://biosciences.dupont.com/); Danisco; Copenhagen, Denmark (https://www.dupontnutritionandbiosciences.com/); DSM, Netherlands (https://www.dsm.com/corporate/home.html); Associated British Foods plc London, UK (http://www.abf.co.uk/), and Takabio, France (https://www.takabio.com/en/). Among them, most of the companies provide xylanases for pulp bleaching; only a handful of commercial xylanases are in the market that can be used in food. Xylanase produced by Novo Nordisk, Denmark, has been found suitable for the brewing industry. Sanzyme X and Albazyme-10A are the names of commercially produced xylanases which are used in the baking industry. A total of 11 endo-xylanases have been commercialized by Megazyme, USA, that has wide applications in the food industry (Table 16.1). Three different xylanases (Panzea® BG, Panzea® 10X BG, and Panzea® Dual BG) are being commercialized by Novozymes for improving the nutritional and techno-physical properties of bread. Novozymes further provides a range of other commercial enzymes for improving the specific property of bread such as Novozymes Lipopan (for stronger dough), Novozymes Gluzym (for stronger gluten), and Novozymes Novamyl (for bread freshness). There are several ventures that provide xylanase with various brand names which cover the multiple facets of the food industry.

16.13 Conclusions and Future Perspectives

Enzymatic food processing is environmentally sustainable because of their low environmental footprint in comparison to chemical-based methods. As compared to chemical counterparts, enzymes are used in very low concentration to yield highly

specific products. Fungal xylanases are gaining momentum in food biotechnology where they are mainly used for modifying food products, rather than the complete degradation of hemicellulosic portion to xylose. In baking industry, xylanase is preferred as dough strengtheners instead of chemical dough conditioners, while in juice and wine industries, they aid in improving the taste, texture, and aroma. Xylanases must, however, have the desirable characteristics that aid in withstanding the extreme process conditions in the downstream processing. Food industries prefer thermostable and acidic xylanases with fair stabilities under operational conditions. Investigations are, therefore, called for filling the legitimate gap to find the ideal biocatalysts for such industries. Majority of acidic xylanases of fungal origin do not fulfill these criteria, thus necessitating search for more from diverse fungal sources.

Prebiotic nature of XOs is well-known by promoting the growth of commensal bacteria in the human gut. Applicability of XOs derived from lignocellulosics by endoxylanase action led to the production of organic acids and elevated levels of commensal bacteria along with a decrease in pathogen count in the gut. Very little is known about the mechanisms involved in modulating the gut microbiota, which need further investigation. Xylan is the second most copious polysaccharide after cellulose, which needs digestion for enhancing the nutritional value. Synergetic action of multiple enzymes is usually required to generate constituent sugars from xylan. The carbohydrate-active enzyme (CAZy) database categorizes various xylanolytic enzymes into 20 different families of glycosyl hydrolases from bacteria, archaea, and eukarya. Bacteria contribute to the major chunk of xylanases, approximately 3847. Prokaryotic xylanases are preferred over those from eukaryotic microbes because the former offer a broader range of xylanases with high substrate specificity, broad spectrum of pH and temperature optima, and fair stabilities at extreme pH and temperatures. These features call for more research on fungal xylanases. Several companies have commercialized xylanases for various applications in food processing industries, where most of them are of fungal origin. Besides traditional cultivation and advanced metagenomic techniques, DNA manipulations (DNA shuffling, directed evolution, and site-directed mutagenesis) can be used to generate improved xylanases with the desired characteristics. Food and healthcare industries can attempt harnessing the beneficial aspects of xylan-hydrolyzing enzymes in order to improve overall quality of products.

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References

1. Arranz-Otaegui A, Carretero LG et al (2018) Archaeobotanical evidence reveals the origins of bread 14,400 years ago in northeastern Jordan. Proc Natl Acad Sci U S A 115:7925–7930
2. Diamond J (2002) Evolution, consequences and future of plant and animal domestication. Nature 418:700–707
3. Dubreuil L, Nadel D (2015) The development of plant food processing in the Levant: insights from use-wear analysis of early epipalaeolithic ground stone tools. Philos Trans R Soc B Biol Sci 370(1682):20140357
4. Gurung N, Ray S et al (2013) A broader view: microbial enzymes and their relevance in industries, medicine, and beyond. BioMed Res Int 2013
5. Kirk O, Borchert TV et al (2002) Industrial enzyme applications. Curr Opin Biotechnol 13: 345–351
6. Whitehurst RJ, van Oort M (2009) Enzymes in food technology. Wiley-Blackwell, Singapore, pp 1–368
7. Scheller HV, Ulvskov P (2010) Hemicelluloses. Ann. Rev. Plant Biol 61:263–289
8. Chemical structure of xylans and their interaction in the plant cell walls. Progress in Biotechnology https://agris.fao.org
9. Bhardwaj RL, Pandey S (2011) Juice blends—a way of utilization of under-utilized fruits, vegetables, and spices: a review. Crit Rev Food Sci Nutr 51:563–570. https://doi.org/10.1080/10408391003710654
10. Alagöz D, Varan NE et al (2022) Immobilization of xylanase on differently functionalized silica gel supports for orange juice clarification. Process Biochem 113:270–280
11. Altınel B, Ünal SS (2017) The effects of certain enzymes on the rheology of dough and the quality characteristics of bread prepared from wheat meal. J Food Sci Technol 54:1628–1637
12. Anand A, Kumar V et al (2013) Characteristics of thermostable endoxylanase and β-xylosidase of the extremely thermophilic bacterium *Geobacillus thermodenitrificans* TSAA1 and its applicability in generating xylooligosaccharides and xylose from agro-residues. Extremophiles 17(3):357–366
13. Kubicek CP (2020) Application of Trichoderma enzymes in the food and feed industries. Trichoderma and Gliocladium 2:341–356
14. Awalgaonkar G, Sarkar S et al (2015) Xylanase as a processing aid for papads, an Indian traditional food based on black gram. LWT Food Sci Technol 62:1148–1153
15. Ayyappan P, Abirami A et al (2016) Physicochemical properties of cookies enriched with xylooligosaccharides. Food Sci Technol Int 22:420–428
16. Bala A, Singh B (2017) Concomitant production of cellulase and xylanase by thermophilic mould *Sporotrichum thermophile* in solid state fermentation and their applicability in bread making. World J Microbiol Biotechnol 33:1–10
17. de Vries RP, Vankuyk PA et al (2002) The *Aspergillus niger* faeB gene encodes a second feruloyl esterase involved in pectin and xylan degradation and is specifically induced in the presence of aromatic compounds. Biochem J 363(2):377–386
18. Garg G, Singh A, Kaur A et al (2016) Microbial pectinases: an ecofriendly tool of nature for industries. 3 Biotech 6:1–13
19. Gowdhaman D, Ponnusami V (2015) Production and optimization of xylooligosaccharides from corncob by *Bacillus aerophilus* KGJ2 xylanase and its antioxidant potential. Int J Biol Macromol 79:595–600
20. Poutanen K (1997) Enzymes: an important tool in the improvement of the quality of cereal foods. Trends Food Sci Technol 8:300–306
21. Raweesri P, Riangrungrojana P et al (2008) α-L-Arabinofuranosidase from *Streptomyces* sp. PC22: purification, characterization and its synergistic action with xylanolytic enzymes in the degradation of xylan and agricultural residues. Bioresour Technol 99:8981–8986

22. Mitreva-Dautova M, Roze E et al (2007) A symbiont-independent Endo-1,4-β-xylanase from the plant-parasitic nematode *Meloidogyne incognita*. Mol Plant-Microbe Interact 19:521–529. https://doi.org/10.1094/MPMI-19-0521
23. Marcellin E, Nielsen LK (2018) Advances in analytical tools for high throughput strain engineering. Curr Opin Biotechnol 54:33–40
24. Verma D, Satyanarayana T (2012) Molecular approaches for ameliorating microbial xylanases. Bioresour Technol 117:360–367
25. Bajpai P (2014) Industrial applications of xylanases. Xylanolytic Enzymes:69–104
26. Eneyskaya EV, Brumer H, Backinowsky LV, Ivanen DR, Kulminskaya AA et al (2003) Enzymatic synthesis of β-xylanase substrates: transglycosylation reactions of the β-xylosidase from *Aspergillus* sp. Carbohydr Res 338:313–325
27. Kim YW, Fox DT et al (2006) Glycosynthase-based synthesis of xylo-oligosaccharides using an engineered retaining xylanase from *Cellulomonas fimi*. Org Biomol Chem 4(10): 2025–2032
28. Rozie H, Somers W et al (1992) Crosslinked xylan as an affinity adsorbent for endo-xylanases. Carbohydr Polym 17:19–28
29. Wong KK, Tan LU et al (1988) Multiplicity of beta-1,4-xylanase in microorganisms: functions and applications. Microbiol Rev 52(3):305–317
30. Dobberstein J, Emeis CC (1991) Purification and characterization of β-xylosidase from *Aureobasidium pullulans*. Appl Microbiol Biotechnol 35(2):210–215
31. Zimmermann W, Winter B et al (1988) Xylanolytic enzyme activities produced by mesophilic and thermophilic actinomycetes grown on graminaceous xylan and lignocellulose. FEMS Microbiol Lett 55:181–185
32. Tamburini E, Costa S et al (2015) Optimized production of xylitol from xylose using a hyper-acidophilic *Candida tropicalis*. Biomol Ther 5:1979–1989
33. Gilbert HJ (2010) The biochemistry and structural biology of plant Cell Wall deconstruction. Plant Physiol 153:444–455
34. Gilbert HJ, Stålbrand H et al (2008) How the walls come crumbling down: recent structural biochemistry of plant polysaccharide degradation. Curr Opin Plant Biol 11:338–348
35. Rogowski A, Baslé A et al (2014) Evidence that GH115 α-Glucuronidase activity, which is required to degrade plant biomass, is dependent on conformational flexibility. J Biol Chem 289:53–64
36. Kumar V, Verma D, Satyanarayana T (2013) Extremophilic bacterial xylanases: production, characteristics and applications. Curr Biotechnol 2:380–399
37. Kaji A, Saheki T (1975) Endo-arabinanase from Bacillus subtilis F-11. Biochim et Biophys Acta (BBA) - Enzymol 410:354–360
38. Kurakake M, Kanbara Y et al (2014) Characteristics of α-L-arabinofuranosidase from *Streptomyces* sp I10-1 for production of L-arabinose from corn hull arabinoxylan. Appl Biochem Biotechnol 172:2650–2660
39. Xu Z, Li S et al (2014) L-arabinose isomerase and its use for biotechnological production of rare sugars. Appl Microbiol Biotechnol 98:8869–8878
40. Rennie EA, Scheller HV (2014) Xylan biosynthesis. Curr Opin Biotechnol 26:100–107
41. Nakamura AM, Nascimento AS et al (2017) Structural diversity of carbohydrate esterases. Biotechnol Res Innov 1:35–51
42. Adesioye FA, Makhalanyane TP et al (2016) Phylogeny, classification and metagenomic bioprospecting of microbial acetyl xylan esterases. Enzym Microb Technol 93–94:79–91
43. Zhang J, Siika-Aho M et al (2011) The role of acetyl xylan esterase in the solubilization of xylan and enzymatic hydrolysis of wheat straw and giant reed. Biotechnol Biofuels 4(1):1–10
44. Wainø M, Ingvorsen K (2003) Production of β-xylanase and β-xylosidase by the extremely halophilic archaeon *Halorhabdus utahensis*. Extremophiles 7:87–93
45. Winterhalter C, Liebl W (1995) Two extremely thermostable xylanases of the Hyperthermophilic bacterium *Thermotoga maritima* MSB8. Appl Environ Microbiol 61:1810–1815

46. Min SY, Kim BG et al (2002) Purification, characterization, and cDNA cloning of xylanase from fungus Trichoderma strain SY. *J Microbiol Biotechnol* 12:890–894
47. Béra-Maillet C, Devillard E et al (2005) Xylanases and carboxymethylcellulases of the rumen protozoa *Polyplastron multivesiculatum Eudiplodinium maggii* and *Entodinium* sp. FEMS Microbiol Lett 244:149–156
48. Jensen JK, Busse-Wicher M et al (2018) Identification of an algal xylan synthase indicates that there is functional orthology between algal and plant cell wall biosynthesis. New Phytol 218: 1049–1060
49. Yamaura I, Koga T et al (1997) Purification and some properties of Endo-l,4-β-d-xylanase from a fresh-water Mollusc, *Pomacea insularus* (de Ordigny). Biosci Biotechnol Biochem 61: 615–620
50. Glass HJ, Stark JR (1995) Carbohydrate digestion in the European lobster *Homarus Gammarus* (L.). J Crustac Biol 15:424–433
51. Bae HJ, Kim HJ et al (2008) Production of a recombinant xylanase in plants and its potential for pulp biobleaching applications. Bioresour Technol 99:3513–3519
52. Saranraj P, Naidu MA (2014) Microbial pectinases: a review. Global J Trad Med Syst. wwwresearchgatenet
53. Harris AD, Ramalingam C (2010) Xylanases and its application in food industry: a review. J Exp Sci 1:1–11
54. Berg P (2008) Asilomar 1975: DNA modification secured. Nature 455(7211):290–291
55. Donohoue PD, Barrangou R et al (2018) Advances in industrial biotechnology using CRISPR-Cas systems. Trends Biotechnol 36:134–146
56. Sanjivkumar M, Silambarasan TS et al (2017) Biosynthesis, purification and characterization of β-1,4-xylanase from a novel mangrove associated actinobacterium *Streptomyces olivaceus* (MSU3) and its applications. Protein Expr Purif 130:1–12
57. Otero DM, Cadaval CL et al (2015) Screening of yeasts capable of producing cellulase-free xylanase. Afr J Biotechnol 14:1961–1969
58. Deswal D, Gupta R et al (2014) Fungal pretreatment improves amenability of lignocellulosic material for its saccharification to sugars. Carbohydr Polym 99:264–269
59. Guan GQ, Zhao PX et al (2016) Production and partial characterization of an alkaline xylanase from a novel fungus *Cladosporium oxysporum*. BioMed Res Int 2016:4575024. https://doi.org/10.1155/2016/4575024
60. Ramanjaneyulu G, Reddy BR (2016) Optimization of xylanase production through response surface methodology by *Fusarium* sp. BVKT R2 isolated from forest soil and its application in saccharification. Front Microbiol 7(SEP):1450
61. Chadha BS, Kaur B et al (2019) Thermostable xylanases from thermophilic fungi and bacteria: current perspective. Bioresour Technol 277:195–203
62. Polizeli MLTM, Rizzatti ACS et al (2005) Xylanases from fungi: properties and industrial applications. Appl Microbiol Biotechnol 67:577–591
63. Maheshwari R, Bharadwaj G et al (2000) Thermophilic fungi: their physiology and enzymes. Microbiol Mol Biol Rev 64:461–488
64. Wang J, Rosell CM et al (2002) Effect of the addition of different fibres on wheat dough performance and bread quality. Food Chem 79:221–226
65. Courtin CM, Delcour JA (2002) Arabinoxylans and endoxylanases in wheat flour bread-making. J Cereal Sci 35:225–243
66. Goesaert H, Slade L et al (2009) Amylases and bread firming – an integrated view. J Cereal Sci 50:345–352
67. Courtin CM, Delcour JA (2001) Relative activity of Endoxylanases towards water-extractable and water-unextractable Arabinoxylan. J Cereal Sci 33(3):301–312
68. Faurot AL, Saulnier L et al (1995) Large scale isolation of water-soluble and water-insoluble pentosans from wheat flour. LWT Food Sci Technol 28:436–441

69. Hoffmann RA, Roza M, Maat J et al (1991) Structural characteristics of the warm-water-soluble arabinoxylans from the tailings of the soft wheat variety Kadet. Carbohydr Polym 16(3):275–289
70. Izydorczyk MS, Biliaderis CG (1995) Cereal arabinoxylans: advances in structure and physicochemical properties. Carbohydr Polym 28(1):33–48
71. Trogh I, Courtin CM et al (2004) The combined use of hull-less barley flour and xylanase as a strategy for wheat/hull-less barley flour breads with increased arabinoxylan and (1→3,1→4)-β-D-glucan levels. J Cereal Sci 40:257–267
72. Delcour JA, van Win H et al (1998) Distribution and structural variation of Arabinoxylans in common wheat mill streams. J Agric Food Chem 47:271–275
73. Peressini D, Sensidoni A (2009) Effect of soluble dietary fibre addition on rheological and breadmaking properties of wheat doughs. J Cereal Sci 49:190–201
74. Martínez-Anaya MA, Jiménez T (1997) Functionality of enzymes that hydrolyse starch and non-starch polysaccharide in breadmaking. Zeitschrift für Lebensmitteluntersuchung und -Forschung A 205:209–214
75. Leys S, de Bondt Y et al (2019) Sensitivity of the *Bacillus subtilis* Xyn a xylanase and its mutants to different xylanase inhibitors determines their activity profile and functionality during bread making. J Agric Food Chem 67:11198–11209
76. Collins T, Gerday C et al (2005) Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol Rev 29:3–23
77. Gebruers K, Brijs K et al (2004) Properties of TAXI-type endoxylanase inhibitors. Biochim Biophys Acta 1696:213–221
78. Juge N, Payan F et al (2004) XIP-I, a xylanase inhibitor protein from wheat: a novel protein function. Biochim Biophys Acta 1696(2):203–211
79. Flatman R, McLauchlan WR et al (2002) Interactions defining the specificity between fungal xylanases and the xylanase-inhibiting protein XIP-I from wheat. Biochem J 365(Pt 3):773–781
80. Gebruers K, Debyser W et al (2001) *Triticum aestivum* L. endoxylanase inhibitor (TAXI) consists of two inhibitors, TAXI I and TAXI II, with different specificities. Biochem J 353 (Pt 2):239
81. Joye IJ, Lagrain B et al (2009) Use of chemical redox agents and exogenous enzymes to modify the protein network during breadmaking – a review. J Cereal Sci 50:11–21
82. Almeida EL, Chang YK (2012) Effect of the addition of enzymes on the quality of frozen pre-baked French bread substituted with whole wheat flour. LWT 49:64–72
83. Katina K, Salmenkallio-Marttila M et al (2006) Effects of sourdough and enzymes on staling of high-fibre wheat bread. LWT Food Sci Technol 39:479–491
84. Stojceska V, Ainsworth P (2008) The effect of different enzymes on the quality of high-fibre enriched brewer's spent grain breads. Food Chem 110(4):865–872
85. Oliveira DS, Telis-Romero J et al (2014) Effect of a *Thermoascus aurantiacus* thermostable enzyme cocktail on wheat bread qualitiy. Food Chem 143:139–146
86. Liu W, Brennan MA et al (2017) Effect of cellulase, xylanase and α-amylase combinations on the rheological properties of Chinese steamed bread dough enriched in wheat bran. Food Chem 234:93–102
87. Liu X, Yan Q et al (2022) Biochemical characterization of a novel glycoside hydrolase family 11 xylanase from *Chaetomium* sp. suitable for bread making. Process Biochem 117:1–9
88. O'Shea N, Kilcawley KN et al (2016) Influence of α-amylase and xylanase on the chemical, physical and volatile compound properties of wheat bread supplemented with wholegrain barley flour. Eur Food Res Technol 242:1503–1514
89. Yegin S, Altinel B et al (2018) A novel extremophilic xylanase produced on wheat bran from *Aureobasidium pullulans* NRRL Y-2311-1: effects on dough rheology and bread quality. Food Hydrocoll 81:389–397
90. Ahmad Z, Butt MS et al (2014) Effect of *Aspergillus niger* xylanase on dough characteristics and bread quality attributes. J Food Sci Technol 51:2445–2453

91. Leys S, Pauly A et al (2016) Modification of the secondary binding site of xylanases illustrates the impact of substrate selectivity on bread making. J Agric Food Chem 64:5400–5409
92. Malik VS, Hu FB (2019) Sugar-sweetened beverages and Cardiometabolic health: an update of the evidence. Nutrients 11:1840
93. Crowe-White K, Parrott JS et al (2017) Metabolic impact of 100% fruit juice consumption on antioxidant/oxidant status and lipid profiles of adults: an evidence-based review. Crit Rev Food Sci Nutr 57:152–162
94. Liska DA, Kelley M et al (2019) 100% fruit juice and dental health: a systematic review of the literature. *Frontiers*. Public Health 7(JUN):190
95. Pepin A, Stanhope KL et al (2019) Are fruit juices healthier than sugar-sweetened beverages? A Review. *Nutrients* 11:1006
96. Siebert KJ (2009) Chapter 2 Haze in Beverages. Adv Food Nutr Res 57:53–86
97. Pinelo M, Zeuner B et al (2010) Juice clarification by protease and pectinase treatments indicates new roles of pectin and protein in cherry juice turbidity. Food Bioprod Process 88: 259–265
98. Adiguzel G, Faiz O et al (2019) A novel endo-β-1,4-xylanase from *Pediococcus acidilactici* GC25; purification, characterization and application in clarification of fruit juices. Int J Biol Macromol 129:571–578
99. Suryawanshi RK, Jana UK et al (2019) Immobilization *of Aspergillus quadrilineatus* RSNK-1 multi-enzymatic system for fruit juice treatment and mannooligosaccharide generation. Food Chem 289:95–102
100. Dhiman SS, Garg G et al (2011) Characterization of statistically produced xylanase for enrichment of fruit juice clarification process. New Biotechnol 28:746–755
101. Kumar L, Nagar S et al (2014) Immobilization of xylanase purified from *Bacillus pumilus* VLK-1 and its application in enrichment of orange and grap juices. J Food Sci Technol 51: 1737–1749
102. Rosmine E, Sainjan NC et al (2017) Statistical optimisation of xylanase production by estuarine *Streptomyces* sp. and its application in clarification of fruit juice. J Genet Eng Biotechnol 15:393–401
103. Bajaj BK, Manhas K (2012) Production and characterization of xylanase from *Bacillus licheniformis* P11(C) with potential for fruit juice and bakery industry. Biocatal Agric Biotechnol 1:330–337
104. Jiang L, Hua D et al (2010) Aqueous enzymatic extraction of peanut oil and protein hydrolysates. Food Bioprod Process 88:233–238
105. Revilla I, González-SanJosé ML (1998) Methanol release during fermentation of red grapes treated with pectolytic enzymes. Food Chem 63:307–312
106. Joutei KA, Chahdi FO et al (2003) Electronic microscopy examination of the influence of purified enzymatic activities on grape skin cell wall. OENO One 37:23–30
107. Nagai M, Ozawa A et al (2000) Purification and characterization of acid-stable protopectinase produced by *aspergillus awamori* in solid-state fermentation. Biosci Biotechnol Biochem 64: 1337–1344
108. Lin J, Massonnet M et al (2019) The genetic basis of grape and wine aroma. Horticul Res 6:81
109. Shin KS, Lee JH (2019) Acetaldehyde contents and quality characteristics of commercial alcoholic beverages. Food Sci Biotechnol 28(4):1027–1036
110. Tavernini L, Ottone C et al (2020) Entrapment of enzyme aggregates in chitosan beads for aroma release in white wines. Int J Biol Macromol 154:1082–1090
111. Wu J, Johnson LA et al (2009) Demulsification of oil-rich emulsion from enzyme-assisted aqueous extraction of extruded soybean flakes. Bioresour Technol 100:527–533
112. Dickey LC, Kurantz MJ et al (2008) Oil separation from wet-milled corn germ dispersions by aqueous oil extraction and aqueous enzymatic oil extraction. Ind Crop Prod 27(3):303–307
113. Aachary AA, Prapulla SG (2011) Xylooligosaccharides (XOS) as an emerging prebiotic: microbial synthesis, utilization, structural characterization, bioactive properties, and applications. Compr Rev Food Sci Food Saf 10:2–16

114. Jain I, Kumar V et al (2015) Xylooligosaccharides: an economical prebiotic from agroresidues and their health benefits. Indian J Exp Biol 53:131–142
115. Otieno DO, Ahring BK (2012) A thermochemical pretreatment process to produce xylooligosaccharides (XOS), arabinooligosaccharides (AOS) and mannooligosaccharides (MOS) from lignocellulosic biomasses. Bioresour Technol 112:285–292
116. Yang R, Xu S et al (2005) Aqueous extraction of corncob xylan and production of xylooligosaccharides. LWT Food Sci Technol 38:677–682
117. Verma D, Satyanarayana T (2012) Cloning, expression and applicability of thermo-alkali-stable xylanase of *Geobacillus thermoleovorans* in generating xylooligosaccharides from agro-residues. Bioresour Technol 107:333–338
118. Verma D, Anand A et al (2013) Thermostable and alkalistable endoxylanase of the extremely thermophilic bacterium *Geobacillus thermodenitrificans* TSAA1: cloning, expression, characteristics and its applicability in generating xylooligosaccharides and fermentable sugars. Appl Biochem Biotechnol 170:119–130
119. Kumar V, Satyanaana T (2014) Production of thermo-alkali-stable xylanase by a novel polyextremophilic *Bacillus halodurans* TSEV1 in cane molasses medium and its applicability in making whole wheat bread. Bioprocess Biosyst Eng 37:1043–1053
120. Chapla D, Pandit P et al (2012) Production of xylooligosaccharides from corncob xylan by fungal xylanase and their utilization by probiotics. Bioresour Technol 115:215–221
121. Reddy SS, Krishnan C (2015) Production of xylooligosaccharides in SSF by *Bacillus subtilis* KCX006 producing β-xylosidase-free endo-xylanase and multiple xylan debranching enzymes.Preparative Bioche. Biotechnol 46(1):49–55
122. Kawaguti HY, Manrich E et al (2006) Production of isomaltulose using *Erwinia* sp. D12 cells: culture medium optimization and cell immobilization in alginate. Biochem Eng J 29:270–277
123. Romero-Fernández M, Moreno-Perez S et al (2018) Designing continuous flow reaction of xylan hydrolysis for xylooligosaccharides production in packed-bed reactors using xylanase immobilized on methacrylic polymer-based supports. Bioresour Technol 266:249–258
124. Gonçalves TA, Damásio ARL et al (2012) Functional characterization and synergic action of fungal xylanase and arabinofuranosidase for production of xylooligosaccharides. Bioresour Technol 119:293–299
125. Liu MQ, Dai XJ et al (2014) Immobilization of *Aspergillus niger* xylanase a on Fe3O4-coated chitosan magnetic nanoparticles for xylooligosaccharide preparation. Catal Commun 55:6–10
126. de Oliveira Nascimento CE, de Oliveira Simões LC et al (2022) Application of a recombinant GH10 endoxylanase from *Thermoascus aurantiacus* for xylooligosaccharide production from sugarcane bagasse and probiotic bacterial growth. J Biotechnol 347:1–8
127. Samanta AK, Kolte AP et al (2016) Value addition of corn husks through enzymatic production of xylooligosaccharides. Braz Arch Biol Technol 59:1–8
128. Ismail SA, Nour SA et al (2022) Valorization of corn cobs for xylanase production by *Aspergillus flavus* AW1 and its application in the production of antioxidant oligosaccharides and removal of food stain. Biocatal Agric Biotechnol 41:102311
129. Yang H, Wang K et al (2011) Production of xylooligosaccharides by xylanase from *Pichia stipitis* based on xylan preparation from triploid Populas tomentosa. Bioresour Technol 102: 7171–7176
130. Dilokpimol A, Nakai H et al (2011) Enzymatic synthesis of β-xylosyl-oligosaccharides by transxylosylation using two β-xylosidases of glycoside hydrolase family 3 from *Aspergillus nidulans* FGSC A4. Carbohydr Res 346:421–429
131. Kurakake M, Fujii T et al (2005) Characteristics of transxylosylation by β-xylosidase from *aspergillus awamori* K4. Biochim Biophys Acta Gen Subj 1726:272–279
132. Yang X, Shi P et al (2014) Two xylose-tolerant GH43 bifunctional β-xylosidase/α-arabinosidases and one GH11 xylanase from *Humicola insolens* and their synergy in the degradation of xylan. Food Chem 148:381–387

133. Cintra LC, Fernandes AG et al (2017) Characterization of a recombinant xylose tolerant β-xylosidase from *Humicola grisea* var. thermoidea and its use in sugarcane bagasse hydrolysis. Int J Biol Macromol 105:262–271
134. Pozo-Rodríguez A, Méndez-Líter JA et al (2022) A fungal versatile GH10 endoxylanase and its glycosynthase variant: synthesis of xylooligosaccharides and glycosides of bioactive phenolic compounds. Int J Mol Sci *23*:1383
135. Walia A, Guleria S et al (2017) Microbial xylanases and their industrial application in pulp and paper biobleaching: a review. 3 Biotech 7:1–12
136. Christakopoulos P, Katapodis P et al (2003) Antimicrobial activity of acidic xylo-oligosaccharides produced by family 10 and 11 endoxylanases. Int J Biol Macromol 31: 171–175
137. Xavier FD, Bezerra GS et al (2018) Evaluation of the simultaneous production of xylitol and ethanol from sisal fiber. Biomol Ther 8:2. https://doi.org/10.3390/biom8010002
138. Dasgupta D, Bandhu S et al (2017) Challenges and prospects of xylitol production with whole cell bio-catalysis: a review. Microbiol Res 197:9–21
139. Pariza MW, Johnson EA (2001) Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Regul Toxicol Pharmacol 33(2):173–186
140. Ladics GS, Sewalt V (2018) Industrial microbial enzyme safety: what does the weight-of-evidence indicate? Regul Toxicol Pharmacol 98:151–154
141. Sewalt V, Shanahan D et al (2016) The generally recognized as safe (GRAS) process for industrial microbial enzymes. Ind Biotechnol 12:295–302
142. Olempska-Beer ZS, Merker RI et al (2006) Food-processing enzymes from recombinant microorganisms—a review. Regul Toxicol Pharmacol 45:144–158

Fungal Therapeutic Enzymes: Utility in the Treatment of Human Ailments

17

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Abstract

This chapter provides an overview of fungal therapeutic enzymes and advancements in applications of these biopharmaceuticals for the treatment of a variety of diseases. Due to their high specificity and catalytic efficiency, enzymes have been frequently used in medicine. Fungal therapeutic enzymes are used to treat a variety of ailments including indigestion, leukemia, pancreatic problems, infectious disease, and generation of prebiotics. Among these, L-asparaginase, for example, has been utilized in the treatment of leukemia because of its anticancer effects. Fungal sources now produce more than half of all the commercial enzymes, including therapeutic biocatalysts, as the fungi-based production is economically viable because of high yields and ease of bioprocess.

Keywords

Fungal enzymes · Therapeutic applications · L-Asparaginase · Amylase · β-Galactosidase · Lipase

17.1 Introduction

Enzymes play a major role in the pharmaceutical industry. Biochemical reactions catalyzed by enzymes help in mitigating diseases because of their ability to catalyze reactions efficiently and selectively. Enzyme-based medications are increasingly becoming a research focus in recent years due to their significant impact on human

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health. The global market for therapeutic proteins was valued at $7082 million in 2017 and is expected to grow at a CAGR of 5.7% from 2018 to 2024, reaching $10,519 million [1]. Industrial production of these therapeutic enzymes typically involves the fermentation of microorganisms (bacteria, yeasts, fungi, etc.) or the use of genetically engineered microorganisms (GEMs). In recent years, advances in recombinant DNA technology, material science, protein engineering, enzyme immobilization, and nanotechnology have created a powerful platform for the development of enzyme-based medications with a wide range of applications in the treatment of diseases. Enzymes involved in fibrinolysis, cancer treatment, digestive system problems, enzyme replacement therapies, and the treatment of other rare and common disorders are being produced using such platforms. They may be useful in the treatment of a variety of human problems such as cancer treatment with amino acid-degrading enzymes, wound and inflammation treatment with anti-inflammatory enzymes, and improving food digestion with digestive aids. The discoveries and advancements in the production of therapeutic enzymes are more stable and effective. Several methods, including rational design, target therapy, PEGylation, immobilization, glycol-engineering, and others, have been used to improve the half-life, solubility, and efficacy, allowing their better usage.

Therapeutic enzymes are the biocatalysts used to treat such medical conditions and diseases. Enzymes have been used as therapeutic agents since the eighteenth century, when these were first used as a digestive aid. Since then, several therapeutic enzymes including L-asparaginase, L-glutaminase, protease, lipase, and β-galactosidase have found significant use in therapy, diagnostics, and research. Therapeutic enzymes have the extra benefit of binding selectively to their targets which distinguishes them from non-enzymatic medicines. However, these benefits have come with a slew of drawbacks including high production costs, elicitation of an immunological response, and shorter in vivo half-life periods [2]. In addition, finding a new mammalian system or microbes that can be used to produce therapeutic enzymes at a low cost and with fewer side effects is a major challenge in the commercial production of therapeutic enzymes [3, 4]. Therapeutic enzymes have broad variety of usage based on their antimicrobials, anticoagulants, mucolytics, thrombolytic, oncolytic, and metabolic properties [4].

Fungi are known to be one of the finest producers of therapeutic enzymes. Because of their eukaryotic origin, substrate selectivity, and stability under a variety of chemical and physical conditions, fungal therapeutic enzymes have received immense attention in the pharma industry. Owing to their ability to secrete high titers of extracellular enzymes, suitability for solid-state cultivation, and easy recovery, fungi have become a preferred option for production of industrial enzymes [5]. Fungal enzymes involved in cancer treatment, digestive disorders, enzyme replacement therapies, and the treatment of other rare disorders are described in the chapter (Fig. 17.1). The chapter deals with some of the prominent fungal therapeutic enzymes, their production strategies, and applications.

Fig. 17.1 Fungal enzymes used for therapeutic purposes

17.2 Anticancer Enzymes

Cancer, being the cause of almost 1 in every 6 deaths worldwide, is the world's second biggest cause of death, with an anticipated global burden of 19 million new cases and 10 million deaths by 2020. By using existing evidence-based preventative measures, about 30–50% of malignancies can be avoided (https://gco.iarc.fr/today/fact-sheets-cancers). In the last five decades, the field of oncology has witnessed the development of many potential solutions.

In many cases, enzyme-deficient tumor cells cannot synthesize several essential amino acids and also intermediate of multiple biosynthetic pathways for its development, proliferation, and survival [6]. In order to compensate these issues, exogenous amino acid supply becomes necessary to meet protein manufacturing demands while maintaining rapid malignant development. This indicates that if the

concentrations of particular amino acids are reduced, tumor cell proliferation may be hampered. On the other hand, normal cells would remain unaffected because non-essential amino acids can be produced within the cell using key metabolic pathway intermediates by many enzymes [7]. Table 17.1 summarizes some of the prominent microbial therapeutic enzymes and their applications.

17.2.1 L-Asparaginase

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) is of great importance in pharmaceutical and food applications. The enzyme catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia (Fig. 17.2**a**). L-Asparaginases (L-ASNase) are found in a diverse range of plants, animals, and microbes [10, 30]; however, microorganisms remain to be the most important source of L-asparaginase. More importantly, bacterial L-asparaginase is a chemotherapeutic enzyme used to treat acute lymphoblastic leukemia in children. Currently, commercial L-asparaginase formulations are derived from *Escherichia coli* and *Erwinia carotovora*. L-Asparaginases can be found in two distinct forms at two different subcellular locations. It is produced constitutively in the cytoplasm as a homodimeric form (L-ASNase I) with a low affinity for L-asparagine. The homotetrameric form (L-ASNase II) of L-ASNase found in the periplasm has a high affinity that is positively regulated by inducers (cyclic AMP) and anaerobiosis [31, 32]. Due to its ability to limit tumor growth and strong affinity for L-asparagine, the second version has received considerable attention from researchers.

Enzymes derived from bacteria can cause adverse reactions such as a relatively high rate of immunological response, silent hypersensitivity, thrombosis, pancreatitis, hyperglycemia, poor plasma half-life, and hepato-toxicity [33]. To obtain an enzyme with less adverse effects, other microbial sources of L-asparaginase must be identified, such as eukaryotic microorganisms. Therefore, research is being done to identify novel sources of L-asparaginase. Due to their eukaryotic origin, fungal L-asparaginase is a possible alternative [34]. Many species of fungi that produce L-asparaginase in significant amount include *Aspergillus*, *Mucor*, and *Penicillium* spp. [35, 36]. *Trichoderma*, *Cladosporium*, and *Saccharomyces cerevisiae* also produce L-asparaginase [37, 38]. Anticancer properties on different cell lines have been tested using purified L-asparaginase derived from marine *Aspergillus niger* AKV-MKBU [11]. In other study, *A. terreus* producing recombinant L-asparaginase having estimated molecular weight of 42.0 kDa which shared 75% homology with *Aspergillus nomius* and 71% with *A. nidulans* L-asparaginase [10]. A gold nanobiocomposite was developed by immobilizing fungal L-asparaginase from *A. terreus* MTCC 1782 onto gold nanoparticles which showed anticancer activity against lung cancer cell line A549 [39].

The development of synthetic biomaterials that resemble blood cells and blood cell nano-crystallization may reveal the directions in which blood cell membrane-based delivery systems will progress in the future [40]. When compared

Table. 17.1 Microbial therapeutic commercial enzymes and their application

SN	Enzyme	EC number	Application	Source	Trade name	Manufacturer	Reference
1	L-Asparaginase	EC 3.5.1.1	Treatment of ALL, T cell lymphoma, lymphosarcoma	*A. niger*, *A. terreus*, *A. oryzae*, *S. cerevisiae*	PreventASe® Acrylaway®	Novozymes, Denmark DSM, Netherlands	[8–11]
2	L-Glutaminase	EC 3.5.1.2	Treatment of ALL	*A. sojae*, *A. oryzae*, *Cryptococcus* sp.	Protana® UBoost	Novozymes, Denmark	[12, 13]
3	Methioninase	EC 4.4.1.11	Breast cancer, Lewis lung cancer, human colon carcinoma, glioblastoma	*A. niger*, *A. flavipes*, *A. ustus*, *A. parasiticus*, *C. tropicalis*	–	–	[14, 15]
4	α-Amylase	EC 3.2.1.1	Easy digestion	*A. oryzae*, *A. awamori*, *A. kawachii*, *A. niger*, *Penicillium* sp., *Mucor* sp.	Pepfiz®, Unienzyme®, Taka-diastase	Ranbaxy Laboratories Ltd., Torrent Pharmaceuticals Ltd., India	[16, 17]
5	Lipase	EC 3.1.1.3	Digest aids/lipids, dyspepsia, gastrointestinal problems	*R. miehei*, *T. lanuginosus*, *P. expansum*, *P. chrysogenum*, *A. niger*, *Fusarium* sp.	Palatase®, Lipozyme® TL, Novozym® 435	Novozymes, Denmark	[18, 19]
6	β-Galactosidases	EC 3.2.1.23	Treatment of lactose intolerance	*A. niger*, *A. terreus*, *A. oryzae*, *Kluyveromyces lactis*	β-Galactosidase	Megazyme, Ireland	[20, 21]
7	Protease	EC 3.4.21–24, 99	To treat stomach disorders, surgical instruments cleaning	*A. oryzae*, *A. niger*, *A. sojae*, *P. chrysogenum*	Flavourzyme®	Novozymes, Denmark Sigma-Aldrich, USA	[22]

(continued)

Table. 17.1 (continued)

SN	Enzyme	EC number	Application	Source	Trade name	Manufacturer	Reference
8	Collagenase	EC 3.4.24.3	Skin ulcers, burns treatment, Dupuytren's disease, liver cirrhosis	*Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp., *Trichophyton* sp., *Microsporum* sp., *Alternaria* sp.	–	–	[23, 24]
9	β-Mannanases	EC 3.2.1.78	Prebiotic generation	*A. niger*, *Talaromyces leycettanus*, *Trichoderma reesei*, *A. oryzae*, *A. quadrilineatus*, *P. oxalicum*, *R. miehei*,	Gamanase®, E-BMANN Mannaway, Cp-mannanase	Novo Nordisk, Megazyme, Ireland Novozymes, Denmark, PhylloZyme, USA	[25–27]
10	Fructosyltransferase (FTase)	EC 2.4.1.9	Prebiotic generation	*A. niger*, *A. oryzae*, *A. terreus*, *Aureobasidium pullulans*, *A. tamarii*	Pectinex® Ultra SP-L	Novozymes, Denmark	[28]
11	Chitinase	EC 3.2.1.14	Treatment of infections, anticancer	*S. cerevisiae*, *C. albicans*	–	–	[29]

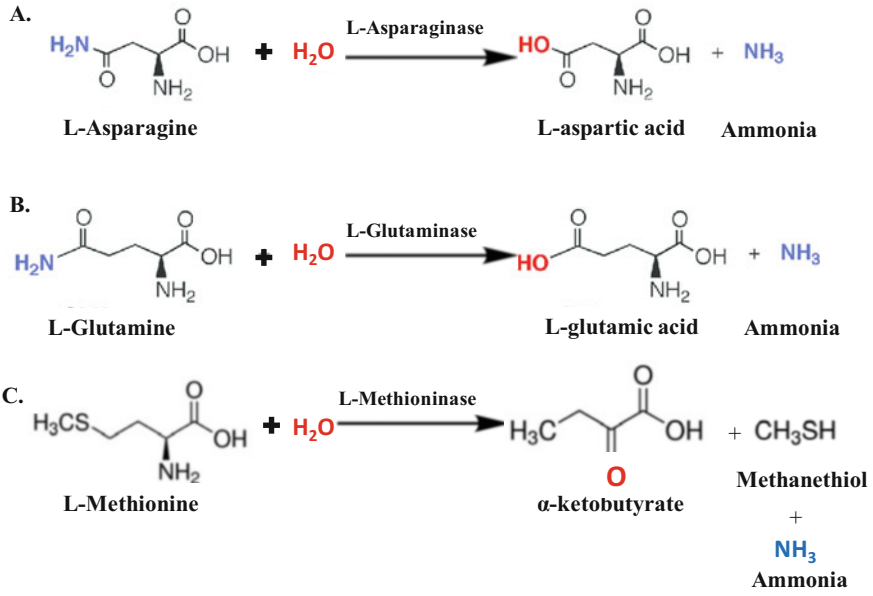

Fig. 17.2 Schematic representation of mechanism of action of therapeutic amidohydrolases: (**a**) L-asparaginase, (**b**) L-glutaminase, and (**c**) L-methioninase

to free or PEGylated L-asparaginase, this approach prevents proteolytic cleavage and reduced antibody recognition, which minimizes the overall dose frequency and immune response risk [41].

L-Asparaginase is used in the food sector in addition to being an anticancer agent. When reducing sugar and the asparagine found in starchy foods react in a Maillard reaction, a serious carcinogen named acrylamide is formed. In a report, L-asparaginase of *A. terreus* was used for the pretreatment of kochchi kesel banana slices before frying to mitigate acrylamide formation during frying. The optimal soaking temperature and time were found to be 60 °C and 20 min, respectively [42].

L-Glutaminase (EC 3.5.1.2) is also an amidohydrolase that catalyzes the conversion of L-glutamine to L-glutamic acid and ammonia in the presence of water (Fig. 17.2**b**) [43]. L-Glutaminases are used as therapeutic agents in cancer therapy, particularly in the treatment of acute lymphocytic leukemia. In vitro tests showed that the treatment was effective against a variety of human cancer cell lines [44]. Apart from its function as a medicinal enzyme, L-glutaminase is also used in the food industries as a flavor enhancer. The catalytic product of glutaminase, i.e., glutamic acid, provides umami (monosodium glutamate) flavor to fermented foods. The SmF technology has been used commercially to produce glutaminase. L-Glutaminase has been produced using submerged cultures of *Aspergillus sojae*, *Aspergillus nidulans*, *Verticillium malthousei*, *Tilachlidium humicola*, and

Penicillium notatum [45]. Few fungal recombinant L-glutaminases such as *A. sojae*, *A. oryzae*, and *Cryptococcus* sp. [12, 13] have also been studied.

The pyridoxal 5′-phosphate (PLP)-dependent enzyme L-Methionine γ-Lyase (MGL, EC 4.4.1.11) catalyzes the conversion of L-methionine to α-ketobutyrate, methanethiol, and ammonia (Fig. 17.2C). Methionine synthase is absent or present in low amounts in methionine-dependent tumor cell lines [46]. However, in healthy cells, it can use betaine and methyl tetrahydrofolate as methyl group donors to synthesize methionine from homocysteine. As a result, dietary methionine deficiency stops tumor cells from dividing in the G2 phase that leads to cell death [15]. MGL is a homo-tetrameric protein that has a molecular weight of about 149–172 kDa, and monomer is about 41–45 kDa [14]. Since it was found to be an effective anticancer agent against a variety of tumor cell lines, including breast, lung, colon, kidney, and glioblastoma, L-methioninase is one of the few microbial enzymes with significant therapeutic efficacy. The MGL has been reported from several fungal species, such as *Aspergillus flavipes*, *A. carneus*, *Penicillium notatum*, *Fusarium solani*, *Geotrichum candidum*, and *Scopulariopsis brevicaulis* [15, 47, 48].

17.3 Enzymes as Digestive Aids

17.3.1 α-Amylase

α-Amylases (EC 3.2.1.1) are predominant glycosyl hydrolases involved in the digestion of carbohydrates that catalyze the conversion of starch to malto-oligosaccharides and dextrin. It is a member of glycoside hydrolase family 13 (GH13), which produces primarily maltodextrins and maltose while maintaining α-anomeric structure in the products by randomly cleaving the α-1, 4 links between adjacent glucose units in starch and related polysaccharides [49]. There are two types of amylases, viz., exo- and endo-amylases. The non-reducing end of starch is hydrolyzed by exo-amylases. Endo-amylases are enzymes that break down glycosidic bonds within the starch molecule [50]. Recombinant fungal amylases, which are active in the dough but inactive during baking, have been identified from mesophilic hosts like *A. oryzae* and are particularly interesting to the food sector since they match the temperature and pH range utilized in applications. The structure and activity of fungal α-amylases from *Cordyceps farinosa*, *Rhizomucor pusillus*, and *Thamnidium elegans*, which have greater pH tolerance and thermostability, have also been reported. The fungal α-amylase has an activity range of pH 4.4 to 6.0 [16, 51]. α-Amylases should be properly chosen in accordance with the requirement if they have properties that are compatible with industrially significant process parameters, such as temperature, pH, substrate type, concentration, and presence of metal ions. Between species and even strains of the same species, purified α-amylase's biochemical characteristics might vary significantly. Amylases were first commercialized in 1984 as a pharmaceutical supplement for the treatment of

digestive diseases. Amylases are also used in fine chemical industries as well as in medical diagnosis [17, 49].

Amylases are used in a variety of sectors including food, alcohol, textiles, detergents, and paper industries [52]. Amylases are most commonly utilized in the starch industry, where they are used for starch hydrolysis in the starch liquefaction and saccharification process generates maltose and malto-oligosaccharides, thus, useful in baking and sugar syrup industries [16]. A wide range of bacterial species secrete thermostable amylases *Bacillus licheniformis*, *B. stearothermophilus*, and *B. amyloliquefaciens* [53]. Fungal amylases or diastases are commercially available as Unienzyme® pills, Pepmax®, Zymtase®, Pepfiz®, and other digestive aids [17]. Several multinational companies (Novozymes, DuPont Danisco, AB Enzymes, Dyadic, BASF, DSM, and others) are engaged in the production of amylases in the commercial sector. Some α-amylase-producing fungal species include genus *Aspergillus* (*A. oryzae*, *A. awamori*, *A. kawachii*), *Penicillium* spp. (*P. brunneum*, *P. expansum*, *P. chrysogenum*, *P. fellutanum*, *P. roqueforti*, *P. camemberti*, *P. janthinellum*, and *P. olsonii*), *Thermomyces lanuginosus*, *Thermomonospora curvata*, *Cryptococcus flavus*, and *Mucor* sp. [16, 21, 54]. In another study, *A. oryzae* IFO-30103 was used to produce α-amylase having molecular weight 51.3 kDa. The purified enzyme showed stability at the pH range of 4.5–7.2 with optimum pH 5.5 [55]. Zhang et al. [56] constructed recombinant α-amylases of *A. niger* after removing *amyR* (encoding a transcription factor) which enhanced the amylase synthesis with low background proteins.

17.3.2 Lipases

Lipases (triacylglycerol hydrolases, E.C. 3.1.1.3) catalyze the hydrolysis of triacylglycerol to glycerol and fatty acids. The versatility of lipases makes them suitable for applications in various sectors including food, dairy, detergents, pharmaceuticals, biodiesel manufacturing, leather, textile, cosmetics, and paper. Because of their catalytic activity, low cost of production, and relative simplicity of genetic manipulation, fungal lipases stand out as one of the most important industrial enzymes [5]. In terms of medicinal relevance, lipases are used to treat dyspepsia, gastrointestinal problems, cutaneous symptoms of digestive allergies, and other infections. Initially, microbial lipases were obtained from *A. flavus* and *Penicillium oxalicum*. Lipases showed pH-dependent activities; usually, they are stable at neutral pH 7.0 or up to pH 4.0 and 8.0. Extracellular lipases were produced by *A. niger* and *Rhizopus* sp., which are active at acidic pH, and alkaline lipases were produced by *Pseudomonas nitroreducens*, which is active at pH 11.0. Lipases are capable of reversing esterification and inter-esterification processes in the absence of water under specific experimental conditions [18, 57]. Although cofactors are not required for the production of lipase activities, Ca^{2+} increases the activity [19, 58]. Co, Ni^{2+}, and Hg^{2+} severely decreased lipase activity, while Zn^{2+}, Mg^{2+}, EDTA, and SDS mildly hindered it. Widely used immobilized lipases are Novozym 435, Lipozyme TL IM, and Lipozyme RM IM sourced from *Candida antarctica*,

T. lanuginosus, and *R. miehei*, respectively [19]. The 3D crystal structures of lipases from *R. miehei* and *Geotrichum candidum* were resolved. Lipases are reported to be monomeric proteins and have a molecular mass in the range of 19–60 kDa [18]. In 1994, Novo Nordisk introduced the first commercial recombinant lipase "Lipolase" which originated from the fungus *Humicola lanuginosa* (*T. lanuginosus*) and was expressed in *A. oryzae*. The major lipase producing genera of filamentous fungi include *Rhizopus*, *Aspergillus*, *Penicillium*, *Mucor*, *Ashbya*, *Geotrichum*, *Beauveria*, *Humicola*, *Rhizomucor*, *Fusarium*, *Acremonium*, *Alternaria*, and *Eurotium* [18, 58]. Commonly used immobilized lipase (Relisorb®) is used in the form of a digestive enzyme cartridge. The medicinal use of lipase improves health of patients by enhancing fat digestion and absorption**.**

17.3.3 β-Galactosidase

The enzyme lactase, also known as β-Galactosidase (β-d-galactoside galactohydrolase, EC 3.2.1.23), hydrolyzes lactose into glucose and galactose and is usually utilized to produce lactose-free milk and other products. β-Galactosidases are extracted from bacteria, fungi, and yeasts [59]. This enzyme is important because it has numerous applications in the food industry, including the creation of lactose-free milk products for persons who are lactose intolerant and the synthesis of glycosylated products. People suffering from lactose intolerance are unable to consume milk and dairy products. Lactose intolerance affects over 70% of the adult population worldwide. Lactose absorption requires the activity of the lactase enzyme, which is present in the small intestine and works by breaking the bond between the two monosaccharides. β-Galactosidases are also used to treat whey and prebiotics [60]. Lactase is sold under a variety of brand names including Lactaid®, Dairy ease®, Silact®, Lacdigest®, etc. β-Galactosidase derived from *A. oryzae* was immobilized under optimal acidic reaction condition and form copper microflower structure [61]. *A. niger* produced 24.64 U/mL of β-galactosidase (76 kDa) [20]. This enzyme is also responsible for the transgalactosylation of lactose to allolactose, which is then split into monosaccharides. Allolactose regulates the quantity of β-galactosidase in the cell by providing positive feedback when it binds to the lacZ repressor.

17.3.4 Proteases

Proteases are a wide class of enzymes that catalyze the breakdown of peptide bonds in proteins. They are also known as peptidases or proteolytic enzymes. Peptide bond cleavage either results in the breakdown of protein substrates into their individual amino acids or, in more specific cases, in the selective cleavage of proteins for post-translational processing and modification. According to the position of the peptide bond that has to be broken, proteases are categorized as peptide hydrolases or peptidases (EC 3.4), which make up a wide family of enzymes. Exopeptidases

(EC 3.4.11–19) and endopeptidases (EC 3.4.21–99) are the two subgroups of proteases. Additionally, they can be divided into three groups based on the pH ranges they prefer: alkaline (pH 8.0 to 13.0), neutral (pH 6.0 to 8.0), and acidic (pH 2.0 to 6.0) [53, 62]. The main source of the acidic protease is a fungus. The uses of alkaline proteases in the food and detergent sectors make them the most significant. The Enzyme Commission has recognized four mechanistic classes, and within these groups, six families of proteases have been identified to date: serine proteases (EC 3.4.21), serine carboxy proteases (EC 3.4.16), cysteine proteases (EC 3.4.22), aspartic proteases (EC 3.4.23), metalloproteases I (EC 3.4.24), and metallocarboxyproteases (EC 3.4.17). In the Global Enzyme Market Report 2018, protease segment alone made one-fourth share of the global enzyme market in 2017 (www.businesswire.com/news/home/20180628006408/en/Global-Enzymes-Market-report-2018).

In numerous physiological and pathological processes, including protein catabolism, blood coagulation, cell growth and migration, tissue organization, morphogenesis during development, inflammation, tumor growth, and metastasis, proteases play a crucial role. These processes also include the release of hormones and pharmacologically active peptides from precursor proteins, the transport of secretory proteins across membranes, and the activation of zymogens [63].

Neto et al. [64] reported novel peptides with antioxidant potential produced from egg and milk proteins hydrolysis with two new fungal proteases isolated from *Eupenicillium javanicum* and *Myceliophthora thermophila*. There have been attempts in recent years to use SmF or SSF to produce various proteases from a variety of substrates. A number of fungal strains have been used to produce proteases belonging to the genera *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor*, *Humicola*, *Thermoascus*, *Thermomyces*, etc.

Different fungal protease genes have been cloned and sequenced using recombinant DNA technology in order to improve their output under ideal circumstances. Genes encoding protease in the fungal strains *T. reesei*, *Aspergillus nidulans*, *A. oryzae*, and *P. chrysogenum* have been expressed heterologously [65]. Fungal proteases are utilized as therapeutic agents to treat a wide range of diseases including diabetes, hepatic malignancy, cancer, HIV, inflammatory diseases, and cancer. Additionally, elastoterase preparation was used to treat severe abscesses, purulent wounds, carbuncles, and furuncles [66]. Clinical therapy that uses collagenolytic proteases directly includes the treatment of retained placenta, sciatica caused by herniated intervertebral discs, and wound healing [23]. In medicine, some fungal potent sources of keratinase may be used in the degrading of keratinized skin, removing hair, preparing a vaccine for dermatophytosis, and increasing the delivery of fungal drugs [67]. Digestive powders are made using fungus aspartic proteases from *A. niger* [68]. In the pharmaceutical sector, alkaline protease is also utilized as a digestible to treat cystic fibrosis. These proteases regulate aberrant bile salt concentrations and pancreatic selections [69]. Since alkaline protease specifically targets fibrin and encourages its destruction, it is employed as an anticancer drug in thrombolytic therapy to treat malignant cells [65]. Human immunodeficiency virus (HIV), which typically targets immune cells, has been treated with protease

inhibitors as a therapeutic treatment. Many protease inhibitors for HIV have received FDA approval. Ritonavir, amprenavir, and indinavir were among these inhibitors. These inhibitors bind to HIV and prevent the virus from replicating [70]. A few protease inhibitors are presently undergoing clinical trials; TMC-114 (Tibotec) is at the clinical phase III stage, and RO033–4649 (Roche) has entered the phase I stage. One of the autoimmune illnesses of the digestive system known as celiac disease affects the small intestine as a result of inadequate digestion of toxic peptides generated from gluten, such as gliadin. Recently, many protease-based treatments have successfully treated celiac disease when combined with a healthy diet and a gluten-free diet [71].

17.4 Anti-inflammatory Enzymes

17.4.1 Collagenase

The fibrous structural protein known as collagen accounts for 30% of all the proteins in the body and is mostly found in the skin, tendons, bones, teeth, blood vessels, intestines, and cartilage. Each collagen molecule is a tiny, rigid stick generated by the interlacing of three alpha chains, or polypeptides, in a triple helix. Collagenases have been shown to have a number of therapeutic benefits in the treatment of burns, intervertebral disc herniation, keloid, cellulite, lipoma, and other disorders. Most widely used commercial source of collagenase is *Clostridium histolyticum*, which is a pathogenic bacterium. Also, collagenase-producing fungi belonging to genera *Aspergillus*, *Cladosporium*, *Penicillium*, *Monacrosporium*, *Trichophyton*, *Microsporum*, and *Alternaria* have been reported. Microbial collagenases are capable of degrading triple-helical collagen and denatured fragments in various sites and are less specific [24, 72]. Collagenases have different applications in the medical field. Collagen re-modeling and cell migration during tissue repair and regeneration is a critical step in the wound healing process where collagenase plays a vital role. These are also used to treat sciatica, in the treatment of a retained placenta, in preclinical therapeutic research for liver cirrhosis, and in the case of accumulation of fibrous plaques in specific body organs (Dupuytren's contracture and Peyronie's disease) [72].

17.5 Anti-infection Enzymes

17.5.1 Chitinases

Chitinases (EC 3.2.1.14) are glycosyl hydrolases that break down β-1,4-glycosidic linkages of chitin [73]. Exo-chitinases facilitate the gradual release of di-acetylchitobiose, while endo-chitinases randomly cleave chitin at interior locations to produce soluble, low molecular oligomers of N-acetylglucosamine. Many harmful organisms, such as fungus, protozoa, and helminths, include chitin

in their cell wall. A chitinolytic enzyme generated from bacteriophage has been used to degrade the cell wall of *Streptococcus pneumoniae*, *Bacillus anthracis*, and *Clostridium perfringens* [74]. Among fungi, chitinases have been reported from *S. cerevisiae* [29] and *T. lanuginosus* [75]. Chitinases can also be used to remove dead skin from burns. Chitinases can be used as potential biomarkers in prognosis and diagnosis of several inflammatory diseases and allergies.

17.6 Enzymes Used in Generating Prebiotics

Prebiotics are nutrients that are selectively utilized by gut bacteria. Prebiotic oligosaccharides have gained popularity as nutraceuticals as a result of growing awareness of the harmful effects of antibiotics and the relevance of prebiotics in modulating gut flora [76]. They can nourish the intestinal flora, and their breakdown products are short-chain fatty acids that reach to the bloodstream impacting not just the gastrointestinal (GI) tract but also distant organs. Fructooligosaccharides (FOS) and mannooligosaccharides (MOS) are two types of prebiotics that have been shown to improve human health. Because oligosaccharides are found in meager amounts in meals, scientists are exploring methods to synthesize prebiotics on a large-scale using microbial enzyme. Prebiotics appear to be promising in boosting human health either as a replacement or in conjunction with probiotics.

17.6.1 β-Mannanase

Endo 1, 4 β-mannanases (EC 3.2.1.78) randomly cleave β-1, 4 glycosidic linkages in the mannan backbone at random, resulting in the formation of various MOS [26, 27]. Mannans are mostly made up of repeated units of mannose joined together by β-1, 4-glycosidic linkages. Mannans are diverse in nature and are classified as glucomannan, galactomannan, or galactoglucomannan based on the presence of glucose in the backbone and/or galactose units as side chains [77]. Recently, MOS from konjac glucomannan, copra meal, and locust bean galactomannan was produced using endo-mannanase from *Aspergillus quadrilineatus*. *Trametes versicolor*, *Pleurotus ostreatus*, *Ceriporiopsis subvermispora*, *Piptoporus*, and *Schizophyllum commune* are examples of white-rot and brown-rot basidiomycetes linked to forest litters and are known to produce mannanolytic enzymes [78]. Prebiotics are widely used in the poultry industry. Examples include commercial preparations like SAF-mannan®, Bio-Mos®, and Active MOS®, which contain α-MOS produced from yeast. Guar gum is hydrolyzed by fungal endo-mannanases into partially hydrolyzed guar gum (PHGG), a heat-tolerant, water-soluble, acid-stable, and taste-free dietary fiber [79]. PHGG ingestion has been proven to improve gut microflora in both animals and humans. These are reported to provide benefits in the treatment of irritable bowel syndrome (IBS) and constipation by growth promotion of probiotic bacteria [80]. Tuohy et al. [81] found that PHGG supplementation increases the population of probiotic bacteria, *Bifidobacterium* and *Lactobacillus*. In

a study, *A. terreus* β-mannanase produced DP3 as predominant oligomer with locust bean gum and guar gum, while DP4 was generated from konjac gum [82].

17.6.2 Fructosyltransferase

Fructosyltransferase (FTase, EC 2.4.1.9) is a major biocatalyst in the production of prebiotic fructooligosaccharides (FOS). It catalyzes the hydrolysis of sucrose molecules as well as the transfer of newly formed fructose residues to acceptor sugar molecules. Fructo-oligosaccharides have bifidogenic, low-calorie, anti-diabetic, anticancer, anti-cariogenic, anti-oxidant, hypolipidemic, and immunomodulatory effects on the host. They also help to improve mineral absorption in the intestines [28]. A number of filamentous fungi and yeasts belonging to *Aspergillus*, *Penicillium*, *Rhizopus*, *Fusarium*, *Xanthophyllomyces*, *Aureobasidium*, *Kluyveromyces*, and *Saccharomyces* have been explored as FTase producers. FTases have also been reported from *A. oryzae*, *A. ibericus*, *A. aculeatus*, and *Aureobasidium pullulans* using SmF [83].

17.6.3 β-Xylanases

Endo-1, 4-D-xylanases (EC 3.2.1.8) are xylanolytic enzymes that cleave the inner 1, 4-xylosidic linkages of the xylan backbone to produce xylooligosaccharides (XOS) [84]. Among glycosyl hydrolase (GH), GH10 and GH11 families of xylanases have received a lot of attention [85].

Xylanases find use in food industry, animal feed, production of biofuels, the pulp and paper bleaching, and the production of XOS [86]. XOS are oligomers consisting of xylose units with a DP ranging from 2 to 6 [87]. It is anticipated that the global market for XOS will increase from USD 93 million in 2017 to USD 130 million in 2023 [84]. Several notable studies demonstrated that XOS had antioxidant and free radical scavenging activity, indicating their potential nutraceuticals [88, 89]. Xylanases from the several fungi such as *A. oryzae*, *Aspergillus kawachii*, *Cryptococcus* sp., and *Penicillium* sp. showed high activity under acidic condition [85, 86, 90, 91].

17.6.4 Xylitol Production

Xylitol is a five-carbon sugar alcohol that occurs in nature and is also a common intermediate in human metabolism. Xylitol prevents enamel demineralization, which lowers acid generation, and has a direct inhibitory impact on mutans streptococci. It also reduces plaque formation and bacterial adherence [92]. These benefits have led to its frequent and consistent use in the food and confectionery sectors [93], and its chemical and nutritional characteristics have been thoroughly examined. The

characteristics of xylitol and its prospective applications as an alternative sweetener are of primary interest.

The yeast *Candida tropicalis* DSM 7524 ferments xylose into xylitol, a nutritive, low-calorie sweetener. The best possibilities for generating xylitol in microbes are yeasts, which use xylose as a possible substrate. In this regard, extensive study has been conducted on a number of yeast strains from the species *C. boidinii*, *C. guilliermondii*, *C. parapsilosis*, and *C. tropicalis* of the genus *Candida* [94], in order to metabolically modify industrial diploid *S. cerevisiae* strains used in the bioethanol sector and further utilize recombinant strains in the synthesis of xylitol in sugarcane straw hydrolysate [95].

17.7 Conclusions

Fungal enzymes have a wide range of applications in the treatment of different diseases including digestive ailments, cancer, infectious diseases, and several others. The production of therapeutic enzymes by fungi is both cost-effective and environmentally benign. The need of the hour is to evaluate novel enzymes from newer strains, as well as to upgrade and improve the existing ones. Isolation, overproduction, and innovative uses of the fungal enzymes in biopharmaceutics should be further investigated to harness their benefits over the conventional chemical therapeutic agents.

References

1. Srivastava A, Srivastava M (2018) Enzymes market type (protease, carbohydrase, lipase, polymerase and nuclease, and other types), source (microorganisms, plants, and animals), reaction type (hydrolase, oxidoreductase, transferase, lyase, and other reaction types), and application–global opportunity analysis and industry forecast, 2017–2024
2. Tandon S, Sharma A, Singh S et al (2021) Therapeutic enzymes: discoveries, production and applications. J Drug Deliv Sci Technol 63:102455. https://doi.org/10.1016/j.jddst.2021.102455
3. Agrawal S, Jana UK, Kango N (2021) Heterologous expression and molecular modelling of L-asparaginase from *Bacillus subtilis* ETMC-2. Int J Biol Macromol 192:28–37. https://doi.org/10.1016/j.ijbiomac.2021.09.186
4. Sabu A (2003) Sources, properties and applications of microbial therapeutic enzymes. Indian J Biotechnol 2:334–341
5. Mehta A, Bodh U, Gupta R (2017) Fungal lipases: a review. J Biotech Res 8:58–77
6. Tabe Y, Lorenzi PL, Konopleva M (2019) Amino acid metabolism in hematologic malignancies and the era of targeted therapy. Blood 134:1014–1023. https://doi.org/10.1182/blood.2019001034
7. Fernandes HS, Silva Teixeira CS, Fernandes PA et al (2017) Amino acid deprivation using enzymes as a targeted therapy for cancer and viral infections. Expert Opin Ther Pat 27:283–297. https://doi.org/10.1080/13543776.2017.1254194
8. Costa IM, Schultz L, de Araujo Bianchi Pedra B et al (2016) Recombinant L-asparaginase 1 from *Saccharomyces cerevisiae*: an allosteric enzyme with antineoplastic activity. Sci Rep 6: 36239. https://doi.org/10.1038/srep36239

9. Freitas M, Souza P, Cardoso S et al (2021) Filamentous fungi producing L-asparaginase with low glutaminase activity isolated from brazilian savanna soil. Pharmaceutics 13(8):1268. https://doi.org/10.3390/pharmaceutics13081268
10. Saeed H, Ali H, Soudan H et al (2018) Molecular cloning, structural modeling and production of recombinant *Aspergillus terreus* L-asparaginase in *Escherichia coli*. Int J Biol Macromol 106:1041–1051. https://doi.org/10.1016/j.ijbiomac.2017.08.110
11. Vala AK, Sachaniya B, Dudhagara D et al (2018) Characterization of L-asparaginase from marine-derived *Aspergillus niger* AKV-MKBU, its antiproliferative activity and bench scale production using industrial waste. Int J Biol Macromol 108:41–46. https://doi.org/10.1016/j.ijbiomac.2017.11.114
12. Ito K, Hanya Y, Koyama Y (2013) Purification and characterization of a glutaminase enzyme accounting for the majority of glutaminase activity in *Aspergillus sojae* under solid-state culture. Appl Microbiol Biotechnol 97:8581–8590. https://doi.org/10.1007/s00253-013-4693-4
13. Masuo N, Ito K, Yoshimune K et al (2004) Molecular cloning, overexpression, and purification of *Micrococcus luteus* K-3-type glutaminase from *Aspergillus oryzae* RIB40. Protein Expr Purif 38:272–278. https://doi.org/10.1016/j.pep.2004.09.003
14. El-Sayed AS, Shouman SA, Nassrat HM (2012) Pharmacokinetics, immunogenicity and anticancer efficiency of *Aspergillus flavipes* l-methioninase. Enzym Microb Technol 51:200–210. https://doi.org/10.1016/j.enzmictec.2012.06.004
15. Khalaf SA, El-Sayed ASA (2009) L-Methioninase production by filamentous fungi: screening and optimization under submerged conditions. Curr Microbiol 58:219–226. https://doi.org/10.1007/s00284-008-9311-9
16. Farooq MA, Ali S, Hassan A et al (2021) Biosynthesis and industrial applications of α-amylase: a review. Arch Microbiol 203:1281–1292. https://doi.org/10.1007/s00203-020-02128-y
17. Vachher M, Sen A, Kapila R, Nigam A (2021) Microbial therapeutic enzymes: a promising area of biopharmaceuticals. Curr Res Biotechnol 3:195–208. https://doi.org/10.1016/j.crbiot.2021.05.006
18. Chandra P, Singh R, Arora PK (2020) Microbial lipases and their industrial applications: a comprehensive review. Microb Cell Fact 19:169
19. Verma S, Meghwanshi GK, Kumar R (2021) Current perspectives for microbial lipases from extremophiles and metagenomics. Biochimie 182:23–36. https://doi.org/10.1016/j.biochi.2020.12.027
20. Martarello RD, Cunha L, Cardoso SL et al (2019) Optimization and partial purification of beta-galactosidase production by *Aspergillus niger* isolated from Brazilian soils using soybean residue. AMB Express 9:81. https://doi.org/10.1186/s13568-019-0805-6
21. Sethi BK, Jana A, Nanda PK et al (2016) Production of α-amylase by *Aspergillus terreus* NCFT 4269.10 using pearl millet and its structural characterization. Front Plant Sci 7:639
22. Banerjee G, Ray AK (2017) Impact of microbial proteases on biotechnological industries. Biotechnol Genet Eng Rev 33:119. https://doi.org/10.1080/02648725.2017.1408256
23. Itou H, Yao M, Watanabe N, Tanaka I (2004) Structure analysis of PH1161 protein, a transcriptional activator TenA homologue from the hyperthermophilic archaeon *Pyrococcus horikoshii*. Acta Crystallogr D Biol Crystallogr 60:1094–1100. https://doi.org/10.1107/S0907444904008522
24. de Albuquerque Wanderley MC, Neto JMWD, de Lima Filho JL et al (2017) Collagenolytic enzymes produced by fungi: a systematic review. Braz J Microbiol 48:13–24. https://doi.org/10.1016/j.bjm.2016.08.001
25. Dawood A, Ma K (2020) Applications of microbial β-Mannanases. Front Bioeng Biotechnol 8:598630. https://doi.org/10.3389/fbioe.2020.598630
26. Kango N, Jana UK, Choukade R (2019) Fungal enzymes: sources and biotechnological applications. In: Satyanarayana T, Deshmukh S, Deshpande M (eds) Advancing frontiers in mycology & mycotechnology. Springer, Singapore, pp 515–538

27. Suryawanshi RK, Jana UK, Prajapati BP, Kango N (2019) Immobilization of Aspergillus quadrilineatus RSNK-1 multi-enzymatic system for fruit juice treatment and mannooligosaccharide generation. Food Chem 289:95–102
28. Choukade R, Kango N (2021) Production, properties, and applications of fructosyltransferase: a current appraisal. Crit Rev Biotechnol 41:1178. https://doi.org/10.1080/07388551.2021.1922352
29. Nagpure A, Choudhary B, Gupta RK (2014) Chitinases: in agriculture and human healthcare. Crit Rev Biotechnol 34(3):215–232. https://doi.org/10.3109/07388551.2013.790874
30. Agrawal S, Sharma I, Prajapati BP et al (2018) Catalytic characteristics and application of L-asparaginase immobilized on aluminum oxide pellets. Int J Biol Macromol 114:504–511. https://doi.org/10.1016/j.ijbiomac.2018.03.081
31. Sajitha S, Vidya J, Karunakaran V, Binod P (2015) Cloning and expression of L-asparaginase from *E. coli* in eukaryotic expression system. Biochem Eng J 102:14–17. https://doi.org/10.1016/j.bej.2015.02.027
32. Shakambari G, Kumar RS, Ashokkumar B et al (2018) Cloning and expression of L-asparaginase from *Bacillus tequilensis* PV9W and therapeutic efficacy of solid lipid particle formulations against cancer. Sci Rep 8:1–11. https://doi.org/10.1038/s41598-018-36161-1
33. Agrawal S, Kango N (2019) Development and catalytic characterization of L-asparaginase nano-bioconjugates. Int J Biol Macromol 135:1142–1150. https://doi.org/10.1016/j.ijbiomac.2019.05.154
34. da Cunha MC, dos Santos Aguilar JG, de Melo RR et al (2019) Fungal L-asparaginase: strategies for production and food applications. Food Res Int 126:108658. https://doi.org/10.1016/j.foodres.2019.108658
35. Gurunathan B, Sahadevan R (2012) Optimization of culture conditions and bench-scale production of L-asparaginase by submerged fermentation of *Aspergillus terreus* MTCC 1782. J Microbiol Biotechnol 22:923–929. https://doi.org/10.4014/jmb.1112.12002
36. Radhika T (2012) Nutritional factors effecting the production of L-asparaginase by the *Fusarium* sp. Afr J Biotechnol 11:3692–3696. https://doi.org/10.5897/ajb10.2355
37. Bon EPS, Carvajal E, Stanbrough M et al (1997) Asparaginase II of *Saccharomyces cerevisiae*. Biotechnol Fuels Chem 63:203–212. https://doi.org/10.1007/978-1-4612-2312-2_19
38. Savitri AN, Azmi W (2003) Microbial L-asparaginase: a potent antitumour enzyme. Indian J Biotechnol 2:184–194
39. Baskar G, Garrick BG, Lalitha K, Chamundeeswari M (2018) Gold nanoparticle mediated delivery of fungal asparaginase against cancer cells. J Drug Deliv Sci Technol 44:498. https://doi.org/10.1016/j.jddst.2018.02.007
40. Bahreini E, Aghaiypour K, Abbasalipourkabir R et al (2014) Preparation and nanoencapsulation of L-asparaginase II in chitosan-tripolyphosphate nanoparticles and *in vitro* release study. Nanoscale Res Lett 9:340. https://doi.org/10.1186/1556-276X-9-340
41. Blackman LD, Varlas S, Arno MC et al (2018) Confinement of therapeutic enzymes in selectively permeable polymer vesicles by polymerization-induced self-assembly (PISA) reduces antibody binding and proteolytic susceptibility. ACS Cent Sci 4:718. https://doi.org/10.1021/acscentsci.8b00168
42. Ravi A, Gurunathan B (2018) Acrylamide mitigation in fried kochchi kesel chips using free and immobilized fungal asparaginase. Food Technol Biotechnol 56(1):51. https://doi.org/10.17113/ftb.56.01.18.5422
43. Bauer K, Bierling R, Kaufman W (1971) Effect of L-glutaminase from *Pseudomonas aureofaciens* in experimental tumors. Naturwissenschaften 58:526–527
44. Patel NY, Baria DM, Yagnik SM et al (2021) Bio-prospecting the future in perspective of amidohydrolase L-glutaminase from marine habitats. Appl Microbiol Biotechnol 105:5325–5340. https://doi.org/10.1007/s00253-021-11416-6
45. Binod P, Sindhu R, Madhavan A et al (2017) Recent developments in L-glutaminase production and applications – an overview. Bioresour Technol 245:1766–1774. https://doi.org/10.1016/j.biortech.2017.05.059

46. Huang KY, Hu HY, Tang YL et al (2015) High-level expression, purification and large-scale production of L-methionine γ-lyase from Idiomarina as a novel anti-leukemic drug. Mar Drugs 13:5492–5507. https://doi.org/10.3390/md13085492
47. Sharma B, Singh S, Kanwar SS (2014) L-Methionase: a therapeutic enzyme to treat malignancies. Biomed Res Int 2014:1. https://doi.org/10.1155/2014/506287
48. Suganya K, Govindan K, Prabha P, Murugan M (2017) An extensive review on L-methioninase and its potential applications. Biocatal Agric Biotechnol 12:104–115. https://doi.org/10.1016/j.bcab.2017.09.009
49. Gupta R, Gigras P, Mohapatra H et al (2003) Microbial α-amylases: a biotechnological perspective. Process Biochem 38:1599–1616. https://doi.org/10.1016/S0032-9592(03)00053-0
50. Saranraj P, Stella D (2013) Fungal amylase-a review. Int J Microbiol Res 4:203–211. https://doi.org/10.5829/idosi.ijmr.2013.4.2.75170
51. Roth C, Moroz OV, Turkenburg JP et al (2019) Structural and functional characterization of three novel fungal amylases with enhanced stability and pH tolerance. Int J Mol Sci 20(19): 4902. https://doi.org/10.3390/ijms20194902
52. Kumar P, Satyanarayana T (2009) Microbial glucoamylases: characteristics and applications. Crit Rev Biotechnol 29(3):225–255. https://doi.org/10.1080/07388550903136076
53. de Souza PM, de Assis Bittencourt ML, Caprara CC et al (2015) A biotechnology perspective of fungal proteases. Braz J Microbiol 46:337–346. https://doi.org/10.1590/S1517-838246220140359
54. Erdal S, Taskin M (2010) Production of α-amylase by *Penicillium expansum* MT-1 in solid-state fermentation using waste loquat (Eriobotrya japonica Lindley) kernels as substrate. Rom Biotechnol Lett 15:5342–5350
55. Chimata MK, Sasidhar P, Challa S (2010) Production of extracellular amylase from agricultural residues by a newly isolated *Aspergillus* species in solid state fermentation. Afr J Biotechnol 9: 5162–5169. https://doi.org/10.5897/AJB10.155
56. Zhang H, Wang S, Zhang XX et al (2016) The amyR-deletion strain of *Aspergillus niger* CICC2462 is a suitable host strain to express secreted protein with a low background. Microb Cell Factories 15:1–11. https://doi.org/10.1186/s12934-016-0463-1
57. Aravindan R, Anbumathi P, Viruthagiri T (2007) Lipase applications in food industry. Indian J Biotechnol 6:141–158
58. Adetunji AI, Olaniran AO (2021) Production strategies and biotechnological relevance of microbial lipases: a review. Braz J Microbiol 52:1257–1269. https://doi.org/10.1007/s42770-021-00503-5
59. Rico-Díaz A, Ramírez-Escudero M, Vizoso-Vázquez Á et al (2017) Structural features of *Aspergillus niger* β-galactosidase define its activity against glycoside linkages. FEBS J 284: 1815–1829. https://doi.org/10.1111/febs.14083
60. Saqib S, Akram A, Halim SA, Tassaduq R (2017) Sources of β-galactosidase and its applications in food industry. 3 Biotech 7:79. https://doi.org/10.1007/s13205-017-0645-5
61. Moreno-Reyes E, Goddard JM (2021) Immobilization of fungal β-galactosidase through self-assembly of hierarchical microflowers under optimal activity conditions. ACS Food Sci Technol 1:304–309. https://doi.org/10.1021/acsfoodscitech.1c00015
62. Sabotič J, Kos J (2012) Microbial and fungal protease inhibitors - current and potential applications. Appl Microbiol Biotechnol 93:1351–1375. https://doi.org/10.1007/s00253-011-3834-x
63. Rao MB, Tanksale AM, Ghatge MS, Deshpande VV (1998) Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62:597–635. https://doi.org/10.1016/S0168-6445(99)00006-6
64. Neto YAAH, Rosa JC, Cabral H (2019) Peptides with antioxidant properties identified from casein, whey, and egg albumin hydrolysates generated by two novel fungal proteases. Prep Biochem Biotechnol 49:639–648. https://doi.org/10.1080/10826068.2019.1566147
65. Naveed M, Nadeem F, Mehmood T et al (2021) Protease—a versatile and ecofriendly biocatalyst with multi-industrial applications: an updated review. Catal Let 151:307–323

66. Gupta R, Beg Q, Lorenz P (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59:15–32. https://doi.org/10.1007/s00253-002-0975-y
67. Gradisar H, Friedrich J, Krizaj I, Jerala R (2005) Similarities and specificities of fungal keratinolytic proteases: comparison of keratinases of *Paecilomyces marquandii* and *Doratomyces microsporus* to some known proteases. Appl Environ Microbiol 71:3420–3426
68. Srilakshmi J, Madhavi J, Lavanya S, Ammani K (2015) Commercial potential of fungal protease: past, present and future prospects. J Pharm Chem Biol Sci 2:218–234
69. Razzaq A, Shamsi S, Ali A et al (2019) Microbial proteases applications. Front Bioeng Biotechnol 7:110
70. Maenéndez-Arias L, Tözsér J (2008) HIV-1 protease inhibitors: effects on HIV-2 replication and resistance. Trends Pharmacol Sci 29:42–49
71. Green PH, Lebwohl B, Greywoode R (2015) Celiac disease. J Allergy Clin Immunol 135:1099–1106
72. Bhagwat PK, Dandge PB (2018) Collagen and collagenolytic proteases: a review. Biocatal Agric Biotechnol 15:43–55. https://doi.org/10.1016/j.bcab.2018.05.005
73. Bhattacharya D, Nagpure A, Gupta RK (2007) Bacterial chitinases: properties and potential. Crit Rev Biotechnol 27:21–28. https://doi.org/10.1080/07388550601168223
74. Mane P, Tale V (2015) Review article overview of microbial therapeutic enzymes. Int J Curr Microbiol Appl Sci 4:17–26
75. Suryawanshi N, Eswari JS (2022) Purification and characterization of chitinase produced by thermophilic fungi *Thermomyces lanuginosus*. Prep Biochem Biotechnol 3:1–9. https://doi.org/10.1080/10826068.2022.2028639
76. Jana UK, Suryawanshi RK, Prajapati BP et al (2021) Prebiotic mannooligosaccharides: Synthesis, characterization and bioactive properties. Food Chem 342:128328
77. Soni H, Kango N (2013) Hemicellulases in lignocellulose biotechnology: recent patents. Recent Pat Biotechnol 7:207–218
78. van Zyl WH, Rose SH, Trollope K, Görgens JF (2010) Fungal β-mannanases: mannan hydrolysis, heterologous production and biotechnological applications. Process Biochem 45:1203–1213
79. Niv E, Halak A, Tiommny E et al (2016) Randomized clinical study: partially hydrolyzed guar gum (PHGG) versus placebo in the treatment of patients with irritable bowel syndrome. Nutr Metab 13(1):10
80. Mudgil D, Barak S, Khatkar BS (2014) Guar gum: processing, properties and food applications—a review. J Food Sci Technol 51:409–418
81. Tuohy KM, Kolida S, Lustenberger AM, Gibson GR (2001) The prebiotic effects of biscuits containing partially hydrolysed guar gum and fructooligosaccharides – a human volunteer study. Br J Nutr 86:341–348
82. Soni H, Rawat HK, Pletschke BI, Kango N (2016) Purification and characterization of β-mannanase from Aspergillus terreus and its applicability in depolymerization of mannans and saccharification of lignocellulosic biomass. 3 Biotech 6(2):136
83. Choukade R, Kango N (2019) Characterization of a mycelial fructosyltransferase from *Aspergillus tamarii* NKRC 1229 for efficient synthesis of fructooligosaccharides. Food Chem 286:434–440
84. Glekas PD, Kalantzi S, Dalios A, Hatzinikolaou DG, Mamma D (2022) Biochemical and thermodynamic studies on a novel thermotolerant GH10 xylanase from *Bacillus safensis*. Biomol Ther 12(6):790. https://doi.org/10.3390/biom12060790
85. Bhardwaj N, Kumar B, Agarwal K et al (2018) Purification and characterization of a thermo-acid/alkali stable xylanases from *Aspergillus oryzae* LC1 and its application in Xylo-oligosaccharides production from lignocellulosic agricultural wastes. Biomacromolecules 122:1191. https://doi.org/10.1016/j.ijbiomac.2018.09.070

86. Ozeki K, Takagi Y, Oda S, Ohashi S (2010) Production of xylanase with a transformant of *Aspergillus oryzae* RIB40 in a liquid-surface immobilization (LSI) system. J Biosci Bioeng 109: 224–226
87. Sluiter A, Hames B, Ruiz R, et al (2008) Determination of structural carbohydrates and lignin in biomass. Laboratory Analytical Procedure, pp 1–16
88. Rashad MM, Mahmoud AE, Nooman MU et al (2016) Production of antioxidant xylooligosaccharides from lignocellulosic materials using *Bacillus amyloliquifaciens* NRRL B-14393 xylanase. J Appl Pharm Sci 6:30–36. https://doi.org/10.7324/JAPS.2016.60606
89. Yu X, Yin J, Li L, Luan C, Li S (2015) Prebiotic potential of xylooligosaccharides derived from corn cobs their *in vitro* antioxidant activity when combined with *lactobacillus*. J Microbiol Biotechnol 25:1084–1092. https://doi.org/10.4014/jmb.1501.01022
90. Knob A, Carmona EC (2010) Purification and characterization of two extracellular xylanases from *Penicillium sclerotiorum*: a novel acidophilic xylanase. Appl Biochem Biotechnol 162: 429–443
91. Krisana A, Rutchadaporn S, Jarupan G et al (2005) Endo-1, 4-betaxylanase B from *Aspergillus cf. niger* BCC14405 isolated in Thailand: purification, characterization and gene isolation. J Biochem Mol Biol 38:17
92. Janakiram C, Deepan Kumar C, Joseph J (2017) Xylitol in preventing dental caries: a systematic review and meta-analyses. J Nat Sci Biol Med 8:16. https://doi.org/10.4103/0976-9668.198344
93. Ahmad I, Shim WY, Jeon WY et al (2012) Enhancement of xylitol production in *Candida tropicalis* by co-expression of two genes involved in pentose phosphate pathway. Bioprocess Biosyst Eng 35:199–204
94. Verma D, Kumar R, Satyanarayana T (2019) Diversity in xylan-degrading prokaryotes and xylanolytic enzymes and their bioprospects. In: Satyanarayana T, Das S, Johri B (eds) Microbial diversity in ecosystem sustainability and biotechnological applications. Springer, Singapore. https://doi.org/10.1007/978-981-13-8487-5_14
95. de Mello FDSB, Maneira C, Suarez FUL et al (2022) Rational engineering of industrial *S. cerevisiae*: towards xylitol production from sugarcane straw. J Genet Eng Biotechnol 20(1):80. https://doi.org/10.1186/s43141-022-00359-8

Heterologous Gene Expression in *Pichia pastoris*: Success Stories and Commercial Ventures

18

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Abstract

Heterologous protein production in the methylotrophic yeast, *Pichia pastoris*, has gained immense popularity owing to the growing demands for recombinant molecules in the health-care and industrial sectors. Its ability to produce correctly folded, bioactive and glycosylated products at high cell densities makes it a desirable expression platform for molecules of therapeutic relevance. Its well-studied genetics and availability of efficient vectors and strong promoters have encouraged researchers to utilize it as an expression platform for large-scale production. Further, genetic manipulation of *P. pastoris* has successfully led to the development of glycoengineered strains that are robust and effective in producing humanized glycoproteins. The *P. pastoris* expression system has many applications, where scientists are constantly working towards the commercialization of its products. Currently, few commercially marketed *Pichia*-based products are used for agricultural, industrial and medical interventions. However, many recombinant products are in the preclinical stage, waiting for regulatory approval. This chapter highlights the current scenario of the protein production system and genetic manipulations based on *P. pastoris*. Further, the chapter discusses its applications and prospects as a leading protein expression platform for enzymes and biopharmaceuticals.

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Keywords

Pichia pastoris · Methylotrophic yeast · Recombinant DNA technology · Signal sequence · High cell density cultivation · Genetic engineering · Biopharmaceuticals · Enzymes

18.1 Introduction

The advent of genetic engineering strategy in 1973 by Herbert Boyer and Stanley Cohen opened the avenues to create functional organisms that contained genetic material from different species. This strategy paved the way to introduce foreign genes into an organism to express and produce recombinant proteins of commercial and therapeutic relevance. Several expression hosts of bacterial, mammalian, plant, insect and yeast origin have been successfully exploited to produce recombinant products. *Escherichia coli* has emerged as a champion organism due to its simple genetics and strategies to develop economical production processes [1]. The bacterial expression system, however, has several bottlenecks like inclusion body formation and the absence of post-translational modification (PTM) mechanisms. Further, the lack of glycosylation and phosphorylation in *E. coli*, which are important for the functional characteristics of a protein, limits its use for therapeutic protein production [2]. Hence, alternate hosts are being explored to overcome the barriers posed in the *E. coli* expression system.

Saccharomyces cerevisiae has developed into a well-characterized eukaryotic yeast expression platform that acts as a model organism for recombinant protein production. It can carry out PTM and secrete proteins into the culture supernatant, thereby simplifying the downstream processing steps. Additionally, it has a remarkable ability to tolerate unfavourable conditions (acidic, high ethanol and sugar concentration) as compared to *E. coli*. Although *S. cerevisiae* has been demonstrated as an efficient host, the methylotrophic yeast, *Pichia pastoris*, has become an efficient cell factory for recombinant protein production due to its effective secretion system [3]. Further, along with their Generally-Regarded-as-Safe status (GRAS), they can carry out PTM, making them suitable hosts for the production of biopharmaceuticals. Several proteins of therapeutic interest like insulin, recombinant human growth hormone (rHGH), anticancer drugs, hormones, food and beverage products, molecules of agricultural importance, detergents, biofuels and vaccines have been successfully expressed in these expression hosts [4–6].

18.1.1 Yeast as a Host for Recombinant Protein Production

Yeasts are widely studied microorganisms whose role in industrial and medical biotechnology is being explored. They possess several distinctive characteristics as they are amenable to genetic modifications, have a comparable fast growth and can be scaled up to the bioreactor level using simple carbon sources. Yeasts have

emerged as a multifaceted system for the economical and high-level production of vaccines as they can express a myriad of antigens. *Saccharomyces cerevisiae* and *Pichia pastoris* have emerged as powerful expression platforms for the development of both animal and human vaccines. Several drug approval agencies, such as Food and Drug Administration (FDA) and European Medicines Agency (EMA) have provided approval for producing cytokines, enzyme substitutes, insulin molecules and vaccines in these yeast cells. The major benefit of using yeast for biotherapeutics production is that they are not contaminated with the virus, thereby eliminating virus testing for molecules synthesized in yeast. For vaccine production, *P. pastoris* has proven to be a better host over *S. cerevisiae* since it possesses a less immunogenic glycosylation pattern and does not cause hyper-glycosylation of proteins. Moreover, high cell density fermentation and specific secretion of recombinant products further reduce the overall production costs [4–6].

18.1.2 *Pichia pastoris*

The methylotrophic yeast, *P. pastoris*, was introduced as a single-cell protein (SCP) for animal feed that later was developed as an expression platform for recombinant proteins. It is widely studied as a model organism for research purposes. The growth of *Pichia* cells in the presence of methanol causes peroxisomes to proliferate, which helps in the study of development, deposits and degradation of organelles participating in the β-oxidation of fatty acids in eukaryotes. It has a simple genetic makeup with an efficient regulation of carbon metabolism. Its ability to metabolize one-carbon compounds makes it an interest of research in biorefinery [7].

The protein expression in *P. pastoris* is initiated under tightly regulated methanol-inducible *AOX* promoter. It shows a preference for respiratory growth, supporting higher cellular biomass. It possesses features like ease of genetic manipulation, higher specific growth and choice of intracellular and extracellular expression with proper post-translational modifications. It can grow to high cell density in minimal media. Moreover, protein-free media lacking endotoxins and viral contamination are used to produce high levels of recombinant molecules. Apart from glycosylation, it carries out proper polypeptide folding, methylation and acylation of recombinant proteins and directs them to different subcellular compartments. Numerous proteins of commercial and therapeutic interest, such as bovine serum albumin, erythropoietin, trypsin, phospholipase C, phytase, isobutanol, α-amylase and antibody fragments, have been expressed using this yeast expression system [8–10].

18.1.3 *Pichia pastoris* vs Other Expression Hosts

The bacterial expression host, *E. coli*, has been widely used since 1980s for protein expression studies. It has a well-defined genetic makeup, shows a higher growth rate and is amenable to genetic modification. However, protein expression in *E. coli* can be demanding under specific conditions. One major issue associated with this

expression system is the formation of insoluble proteins aggregating to form inclusion bodies. Moreover, it lacks post-translational protein modifications, leading to unfavourable expression of eukaryotic proteins. The glycosylation modification is especially critical for therapeutic proteins that should not raise any antigenic immune response when delivered to humans. Even though it can be easily scaled up to a bioreactor level, its high cell density cultivation is limited. Hence, there is a need to move to eukaryotic hosts such as yeast that may overcome the hurdles present in the *E. coli* protein expression platform. *S. cerevisiae* stands as the next desirable eukaryotic host for protein production. Its ability to carry out post-translational modifications and tolerate hostile conditions such as low pH and high sugar concentrations makes it a model organism for recombinant protein expression. Nevertheless, *S. cerevisiae*-based expression system also possesses some drawbacks. It has been observed that the secretion ability of this yeast is limited, resulting in moderate expression yields. Further, it has an unbalanced metabolic system that leads to excessive by-product formation. *S. cerevisiae* adopts a fermentative mode of metabolism and produces ethanol at high cell density cultivation. Although glycosylation is an important modification for the functional aspects of a protein, the glycosylation pattern in *S. cerevisiae* differs from that of mammalian cells. The O-linked glycosylation consists of only mannose residues in this yeast, in contrast to higher eukaryotes, which have O-linked chains with sialic acid residues. In addition, *S. cerevisiae* causes hyper-glycosylation of the N-linked sites, altering the protein binding and functionality. Furthermore, it terminally adds α-1,3 mannose glycan linkages in the core of long oligosaccharides that are hyperallergic and hence not desirable for therapeutic molecules [3, 7, 11–13].

As a result, *P. pastoris* serves as an ideal protein expression host that overcomes the challenges posed by the other two host systems. Apart from sharing its features with mammalian cell lines, such as CHO cells, it can be manipulated in a cost-effective manner. In addition, its fast expression rate and ability to process proteins co- and post-translationally makes it a promising expression system for eukaryotic proteins, and it can also be scaled up to a bioreactor level to obtain high cell densities. Moreover, it has a specific secretion mechanism and secretes very few native proteins into the culture supernatant, thereby reducing the downstream processing costs. It can carry out disulphide bond formation and the target proteins' O- and N-linked glycosylation. *Pichia* may not hyper-glycosylate glycoproteins as it adds short oligosaccharide chains to the proteins. It also lacks immunogenic α-1,3 mannose residues, making it appropriate for biopharmaceutical production. Further, a low degree of O-glycosylation has been observed in *P. pastoris* [12].

P. pastoris has been developed as an effective expression platform and model organism for recombinant products. Recently efforts have been made to engineer its genome to make it more suitable for producing products with humanized glycosylation patterns in high cell density cultivation. This chapter describes the various components of the *P. pastoris*-based expression system, highlighting the commercial ventures undertaken using this methylotrophic yeast.

18.2 *Pichia pastoris* as a Protein Expression Platform

18.2.1 *P. pastoris* as a Methylotrophic Yeast

Apart from *P. pastoris*, a few species of yeast such as *Hansenula* and *Candida* possess the ability to grow on methanol. There was immense research interest on these methylotrophic strains, especially on *P. pastoris*, to produce single-cell protein (SCP) by utilizing methanol as the sole carbon source. The Phillips Petroleum Company was responsible for developing *P. pastoris* fermentation media and protocols used to synthesize SCP from methanol. However, due to the oil crisis in the 1970s, SCP production was deemed commercially unviable, and instead, *P. pastoris* emerged as one of the relevant organisms for biotechnology use. Along with Salk Institute Biotechnology/Industrial Associates Inc. (SIBIA, La Jolla, CA, USA), the Phillips Petroleum Company utilized this yeast to produce recombinant proteins [14]. It has emerged as a successful host for studying heterologous protein expression and peroxisomal biogenesis. The estimated genomic size of *P. pastoris* GS115 is 9.43 Mbp and assembled into four chromosomes containing 5313 coding genes (http://www.pichiagenome.org) [15].

The utilization of methanol as the sole carbon source involves a metabolic pathway comprising several enzymes. Alcohol oxidase (AOX) in the methanol utilization pathway is encoded by two genes, i.e. *AOX1* and *AOX2*. *AOX1* gene is responsible for the significant fraction of the methanol-oxidizing activity, thereby converting methanol to formaldehyde and hydrogen peroxide. The regulation of *AOX1* is carried out at the transcription level by the strong methanol-inducible promoter, P_{AOX1}. The tightly regulated P_{AOX1} promoter is induced by methanol, whereas it undergoes repression in the presence of glucose, glycerol or ethanol [16, 17].

18.2.2 Glycosylation

The most commonly used post-translational modification is glycosylation, which is further divided into N- and O-linked glycosylation. An N-linked glycosylation refers to the addition of $Glc_3Man_9GlcNAc_2$ oligosaccharide to asparagine residue present within the consensus sequence N-X-S/T. The high mannose glycans in *P. pastoris* consist of 9–16 mannose residues. The role of N-glycosylation has been widely studied as it takes part in the signalling of protein folding involving calnexin. The O-linked glycosylation involves the addition of mannose residue to the serine or threonine amino acid residues [18].

18.2.3 Recombinant Protein Production Using *P. pastoris* as Expression Host

The expression of a foreign gene in *P. pastoris* requires the introduction of the gene into an appropriate expression vector with a strong promoter and the insertion of the expression cassette into the host cell genome. The selection of *P. pastoris* strains for recombinant protein expression is also critical for product quantity and quality [12].

18.2.3.1 Expression Vectors

The *P. pastoris* expression vectors comprise (a) promoter sequence located in the 5′ region, (b) transcription terminator sequence in the 3′ region and (c) a multiple cloning site. The episomal vectors are not used in *P. pastoris*; instead, the expression vectors are first linearized and then are allowed to integrate into the host genome. The development of expression cassette is done in *E. coli* with the help of shuttle vectors. Shuttle vectors carry an origin of replication for plasmid propagation in bacteria and have resistance to different agents such as geneticin (*Kan*), zeocin (*Sh ble*), blasticidin (*Bsd*), ampicillin (*Amp*) and formaldehyde (*FLD1*). Further, the secretion of foreign proteins into the extracellular medium is supported by the presence of secretion signals such as *S. cerevisiae* α-mating factor and acid phosphatase (*PHO1*) native to *P. pastoris* [12, 19].

18.2.3.2 Promoters

The *AOX1* promoter of the methanol utilization (MUT) pathway is a strong promoter and hence used in developing several expression vectors. Under the regulation of the *AOX1* promoter, the expression of the heterologous proteins is tightly regulated and maintained by a repression/de-repression system. The biomass production and protein induction phases are separate in *AOX*-regulated expression. A higher biomass is achieved when the *AOX1* promoter is repressed by the presence of carbon sources apart from methanol. Whereas an increased protein expression level is attained when the *AOX1* promoter is induced by methanol. However, the methanol-inducible system has several drawbacks. It is a highly flammable chemical, making its large-scale storage unfavourable. The monitoring of methanol levels is difficult during fermentation. The need for a different biomass and protein production phase makes the mechanism more cumbersome. Further, the use of methanol for the production of food-grade materials is not approved [20].

The promoter of glyceraldehyde-3 phosphate dehydrogenase (*GAP*) in the glycolysis pathway is a strong promoter and utilized for the constitutive expression of recombinant proteins. The *GAP* promoter has several advantages over the inducible *AOX1* system, as it does not require a methanol phase, thereby avoiding methanol-related hazards [21]. The *GAP* promoter has been shown to target the expression of proteins reaching levels similar to that of the *AOX1* promoter. Since it does not require an induction step, the process becomes much simpler and shorter [22].

The *FLD1* (formaldehyde dehydrogenase I) is an alternate promoter similar to the *AOX1*. It is a strong promoter induced either by methanol or methylamine when used as a sole carbon and nitrogen source, respectively. When methylamine is used as an

inducer molecule, it is typically used in conjugation with sorbitol or glucose as a carbon source. Since both the *AOX1* and *FLD1* promoters are induced by methanol, methylamine can be used to induce separately only one of the genes cloned under the *FLD1* promoter. Based on this, Duan and co-workers worked on developing a dual promoter vector system comprising *AOX1* and *FLD1* promoters. The vector was used to target the co-expression of green fluorescent protein (GFP) and gelatin. While gelatin was secreted into the extracellular broth, GFP was expressed intracellularly under the *FLD1* promoter. Moreover, it was reported that induction using 0.5% methanol was sufficient to co-express both the proteins [23].

The promoter of *ICL1* gene coding isocitrate lyase enzyme of glyoxylate cycle has been used as an alternative to express recombinant proteins. The promoter repression occurs when glucose is present, but it is induced in the absence of glucose or the presence of ethanol. Other promoters such as *PEX8* and *YPT1* are moderate in strength and result in low foreign protein expression. This is especially desirable when high-level protein production from strong promoters results in improper folding and subsequent degradation of proteins. The *PEX8* promoter regulates the expression of peroxisomal matrix protein required for the biogenesis of peroxisome. It is expressed at a low level when cells are grown in glucose, but upon shifting to methanol, the expression increases. *YPT1* encodes GTPase whose promoter is used to target low constitutive heterologous protein expression. Several compounds, such as glucose, mannitol or methanol, can be used as suitable carbon sources [20].

Since the inducible promoters in *P. pastoris* are based on the induction caused by particular carbon or nitrogen sources, attempts have been made to design promoters that are not based on growth media components. Under certain circumstances, shifting the yeast cells into a different carbon or nitrogen source may not be favourable for induction. Hence, as a viable solution to this problem, Koller and co-workers developed a vector where the *CUP1* promoter native to *S. cerevisiae* was cloned into pPIC3K, creating the pJV4 vector. The efficiency of this vector was tested by expressing GFP-SKL (green fluorescent protein–tripeptide peroxisomal targeting signal) under the *CUP1* promoter that was induced by the addition of copper [24]. The *ADH3* gene (alcohol dehydrogenase) native to *P. pastoris* is involved in ethanol consumption, and the role of its promoter in recombinant protein expression has been studied. The *ADH3* promoter was targeted to express *Aspergillus niger* xylanase (*XylB*), and its efficacy was highlighted by comparing its expression with the *GAP* and *AOX1* promoters [25, 26].

18.2.3.3 Selection Markers

The selection markers in a vector are required to carry out the genetic manipulation of *P. pastoris*. There are several antibiotic resistance genes, such as *Sh ble* (zeocin), *bsr* (blasticidin), *nptII* or *nptIII* (kanamycin), out of which the *Sh ble* gene is the widely used drug resistance marker native to *Streptoalloteichus hindustanus*. Other commonly used resistance markers include the blasticidin S deaminase (BSD) gene endogenous to *Aspergillus terreus* that imparts resistance to blasticidin [9]. Novel vectors containing resistance for G418 have been created that house a modified *Tn903kan*^{r} gene that allows selection in both bacteria and *P. pastoris*. These vectors

pKAN B and pKANα B are beneficial since they are more economical, efficient, offer high transformation efficiency and have small sizes [27]. The *P. pastoris FLD1* gene encodes the enzyme formaldehyde dehydrogenase. The yeast *fld1* mutants have an enhanced sensitivity to formaldehyde, and its resistance increases with an increase in the number of *FLD1* genes, which can be used as a selection marker. The *FLD1* gene can be used as a marker that allows the selection of transformants with a higher degree of resistance towards formaldehyde [28]. Another drug resistance marker that has been studied is the soraphen resistance gene that imparts resistance against soraphen A, a macrocyclic polyketide native to *Sorangium cellulosum* [29]. The *hph* gene native to *Klebsiella pneumoniae* was placed under the regulation of *S. cerevisiae TEF1* (transcription elongation factor) promoter to construct hygromycin resistant *P. pastoris* expression vectors. The efficacy of this system was tested by carrying out the constitutive or inducible expression of green fluorescent protein (GFP) and human serum albumin (HSA) [30].

Apart from antibiotic resistance genes, biosynthetic markers of *S. cerevisiae* and *P. pastoris* are used as efficient selection markers. Although *S. cerevisiae* genes such as *ARG4* (argininosuccinate lyase) and *HIS4* (histidinol dehydrogenase gene) have been developed as selection markers, genes native to *P. pastoris* have also been explored. The characterization of *P. pastoris ARG4*, *ADE1* and *URA3* genes has shown that these are similar to the *S. cerevisiae* enzymes encoding argininosuccinate lyase, PR-aminoimidazole succinocarboxamide synthase and orotidine-5′--phosphate decarboxylase, respectively. The mutant strains of *P. pastoris*, namely *ade1*, *arg4* and *ura3*, highlight the development of expression systems based on these biosynthetic markers [31]. Screening of *P. pastoris* genome has also helped in characterizing other biosynthetic genes native to *P. pastoris* such as *ARG1*, *ARG2*, *ARG3*, *HIS1*, *HIS2*, *HIS5* and *HIS6*, which have been observed to show homology towards *S. cerevisiae* counterpart genes [32]. The range of *P. pastoris* selection markers have been diversified by the cloning and characterization of *MET2* gene that is homologous to the *S. cerevisiae MET2* gene (*ScMET2*). This gene encoding the enzyme homoserine-*O*-transacetylase was used as a biosynthetic marker to construct the intracellular and extracellular *Pichia* expression system [33].

18.2.3.4 Secretion Signals

The major advantage of using *P. pastoris* as an expression host is that it secretes a high quantity of properly folded proteins into the culture media. The frequently used signal sequences include *S. cerevisiae* α-mating factor (α-MF) and invertase (*SUC2*), as well as its native acid phosphatase (*PHO1*) [34]. The α-MF is a pre-pro-signal peptide consisting of a pre-sequence of 19 amino acid residues, followed by a 67-residue pro-sequence. There are three consensus N-glycosylation targets along with a processing site for dibasic Kex2 endopeptidase. A three-step strategy accounts for the processing of the α-mating factor. The first step involves the removal of the pre-sequence by signal peptidases in the endoplasmic reticulum. This is followed by the action of the Kex2 endopeptidase that cleaves between the arginine and lysine residues and removes the pro-sequence. In the final step, the Ste13 protein causes the rapid cleavage of the Glu-Ala repeats, a phenomenon in the

Golgi apparatus. Although the α-MF is a widely used secretion signal, certain proteins that, when fused to it, are not appropriately processed and get retained in the ER or Golgi. As a result, various strategies such as site-directed mutagenesis has been used to remove particular sets of amino acids in the α-MF and fused to target proteins (horseradish peroxidase and *Candida antarctica* lipase B) to improve the secretion efficiency [35]. A range of alternate signal peptides, including that of *Aspergillus niger* α-amylase, *P. pastoris* Exg1p, *Kluyveromyces marxianus* inulinase, *Gallus gallus* lysozyme, *Homo sapiens* serum albumin and *S. cerevisiae* invertase, has been tested for the efficient secretion of the sweet protein, brazzein [36].

PIR (**P**rotein with **I**nternal **R**epeats) are covalently linked cell wall proteins extensively found in budding yeast such as *S. cerevisiae*, *Yarrowia lipolytica* and *P. pastoris*. Two different PIR proteins Pir1p and Pir2p were isolated from *P. pastoris* genome, out of which Pir1p consists of a pre-pro type of structure with a site for Kex2 protease. A novel expression system was constructed based on the Pir1 signal sequence, where the secretion signal was used to drive the successful extracellular expression of GFP, and therapeutic proteins such as human α-1-antitrypsin (AAT) and streptokinase [37, 38]. Further studies on secretion signals have highlighted the usage of murine IgG1 signal peptide for targeting the secretion of VRC01, a neutralizing antibody against HIV. The better secretion capability of murine IgG1 signal peptide as compared to α-MF offers the possibility of obtaining high yields of a range of antibody molecules [39].

18.2.3.5 Host Strains

The strains of *P. pastoris* used for expression studies are obtained from NRRL-Y 11430 (Northern Regional Research Laboratories, Peoria, IL, USA) or the wild-type *P. pastoris* X-33 strains. The auxotrophic strains such as *arg4*, *his4* and *ura3* and their combinations could be used as selectable markers. The most widely used strain has a mutation in the histidinol dehydrogenase (*HIS4*) that helps in selecting expression vectors containing the *HIS4* gene after transformation. The degradation of recombinant proteins is an undesirable phenomenon that lowers protein yield and activity and hinders downstream processing. As a result, several protease-deficient strains have been developed that are efficient for proteins sensitive to degradation. Some of these strains including SMD1163, SMD1165 and SMD1168 are deficient in proteases such as vacuolar aspartyl protease (*PEP4*), carboxypeptidase Y (*PRC1*) and proteinase B (*PRB1*) [9, 34].

Although protein glycosylation is an important post-translational modification, it may prove to be a concern depending on the applications of the protein. Glycosylation confers modified characteristics and functions to the glycoprotein. A lack of sialic acid residue causes quick removal of the protein from the bloodstream. Hence, studies have focused on engineering *P. pastoris* to humanize N-glycosylation in the organism. Several strategies have been proposed, such as inactivating the *OCH1* gene (α-1,6-mannosyltransferase), which imparts hyper-mannosylation in yeast cells [40].

18.2.3.5.1 PichiaPink Yeast Expression System

PichiaPink™ is an efficient *P. pastoris*-based system that screens transformants expressing high protein titers. These strains have numerous advantages over the already existing *P. pastoris* strains. The major characteristic feature of these strains is that they are auxotrophic for *ADE2* gene that is unable to grow in adenine-deficient media due to the complete and partial deletion of the *ADE2* gene and its promoter, respectively. The *ADE2* participates in the de novo synthesis of purine nucleotides. The commercially available kit contains a vector with a full-length *ADE2* gene under the regulation of its own promoter, which is also employed as a selection marker. Hence, positive transformants regain the ability to grow on a minimal medium as the *ADE* complementation is achieved. Further, this system provides an easy pink/white screening system such that the pink colonies synthesize less quantity of the ADE2 protein, but the white colonies express higher amounts of ADE2 protein, indicating a high number integration of the foreign gene. In addition, three protease-deficient strains of PichiaPink™ are available. These knock-out genes of the proteases are *pep4* (proteinase A), *prb1* (proteinase B) and a double knock-out strain lacking both proteinase A and B. The strong *AOX1* promoter drives the high-level expression of the heterologous proteins. Several therapeutic proteins such as human serum albumin (HSA), *Ganoderma lucidum* immune regulatory protein, lactoferrin and human platelet-derived growth factor-BB have been expressed successfully using the PichiaPink™ system [41–43]. The protease-deficient strains of this system have proven to be fruitful as it was used to surface-display *Rhizomucor miehei* lipase (RML) in *P. pastoris* [44].

18.2.3.5.2 GlycoSwitch®

Biopharmaceuticals that are therapeutically relevant require post-translational modifications (PTM), out of which glycosylation is the most common protein modification. One of the glycol-engineered strains of *P. pastoris* is GlycoSwitch® by BioGrammatics, Inc., which has a deleted *OCH1* gene that helps in mannose chain extension. Further, this strain possesses α-1,2-mannosidase to trim the Man8 to Man5 glycan. Thus, the strain has acquired the ability to produce glycoproteins with a humanized N-type glycosylation pattern [45]. Several therapeutic biomolecules such as L-Asparaginase, antibody Trastuzumab (Herceptin) and human interferon-alpha 2b have been successfully expressed using the glycoengineered strains of *P. pastoris* [45–47].

18.2.3.6 Methanol Utilization (MUT) Phenotypes

There are three different strains of *P. pastoris* depending upon their methanol utilizing ability. In cases where both the *AOX* genes are present within the *Pichia* genome, the strains are referred to as Mut^+ phenotype (methanol utilization plus). This strain is characterized by a higher growth rate than other strains. The second phenotype is the Mut^s strain (methanol utilization slow) that possesses only the *AOX2* gene and has the *AOX1* gene knocked out. Since the *AOX2* gene is regulated by a weak promoter *p*AOX2 and accounts for only 15% of the AOX activity, the growth rate in Mut^s phenotypes is low due to the slow utilization of methanol. The

third phenotype, Mut^- strain, does not contain both the *AOX* genes and hence has lost the ability to grow on methanol. The methanol utilization pathway genes are transcriptionally repressed in Mut^+ and Mut^s cells when grown at a high glucose or glycerol concentration [48].

18.2.3.7 Transformation in *P. pastoris*

Yeast transformation can be carried out using chemical, enzymatic and physical methods. The chemical methods use reagents such as lithium chloride, lithium acetate or polyethylene glycol (PEG). The physical methods include electroporation, which is preferred over the chemical or glass bead method. Spheroplast preparation is the enzyme-based strategy that involves the preparation of spheroplasts, followed by plating on regeneration agar for transformants. Spheroplast generation is a cumbersome process and less efficient, therefore, other alternatives are preferred. Alkali metal ions and detergents are often explored to prepare competent cells. When electroporation and LiCl methods were compared for the transformation of *Candida tropicalis* xylose reductase in *P. pastoris*, it was found that the number of transformants were 1×10^6 and 2×10^3, respectively. The DNA-mediated transformation in *Pichia* depends on several factors such as economics, convenience and size of the expression cassette [49, 50].

P. pastoris expression vectors are integrative, not episomal. The vector DNA is linearized and transformed into the host, which produces stable transformants via homologous recombination between the vector and host DNA sequences. The multicopy integrants are favoured to achieve a higher quantity of the desired product. The in vitro multimerization is another approach to create multiple copies of expression cassettes before transformation to a selected strain. The post-transformational vector amplification (PTVA) is an approach to generate recombinant strains with a genome possessing head-to-tail copies of the vector, integrated at a single locus. Further, vector based on antibiotic resistance gene such as *Sh ble* can be used to configure zeocin resistance. Hence, if the expression cassette has integrated in multiple numbers into the *Pichia* genome, then a higher quantity of the resistance gene product will be produced, thus imparting a higher resistance to the drug. However, such a process is considered to be time-consuming and inefficient [19, 51, 52].

18.3 Genetic Manipulation of *Pichia* for Protein Production

Methylotrophic yeast *P. pastoris* is a widely used expression host to produce recombinant products. Secretion of recombinant proteins, minimum contamination from intrinsic *Pichia* proteins, efficient glycosylation and high cell density cultivation makes it an effective expression platform. In past years, plenty of efforts have been made to understand the physiology, cell biology and genetics of *Pichia*. Nevertheless, the high-level production of recombinant proteins depends on several optimization steps. Thus, to target efficient production, the optimization strategies involve host strain engineering, modulation of cultivation conditions such as media components and physiological growth parameters, alteration in signal peptides and

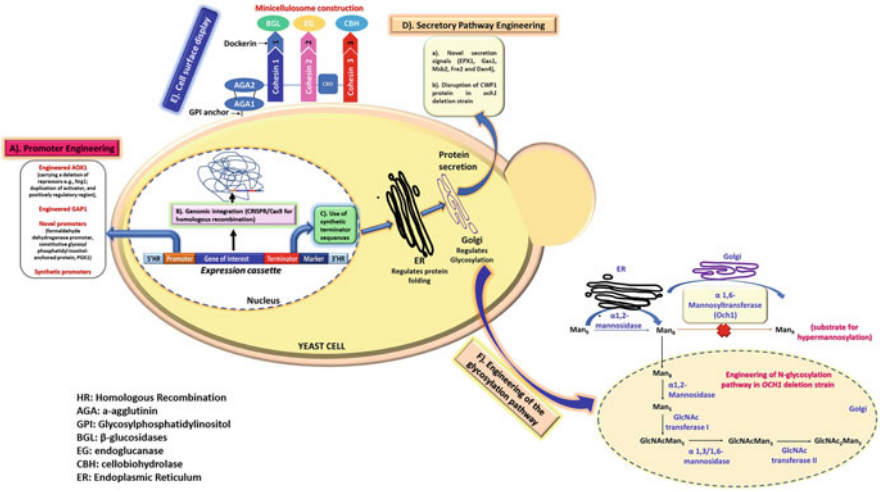

Fig. 18.1 Different strategies for engineering the *Pichia* host system for recombinant protein expression. (**a**) Engineering of promoters; (**b**) different genomic integration strategies for enhancing the efficiency of homologous recombination; (**c**) engineering of terminator sequences; (**d**) secretory pathway engineering; (**e**) cell surface display technology for the construction of minicellulosome and other enzyme systems; (**f**) engineering of N-glycosylation for humanization of the *Pichia* glycosylation pathway

fine-tuning of different expression cassettes. In the following section, various engineering aspects of *Pichia* are discussed in detail (Fig. 18.1).

18.3.1 Engineering of *Pichia* Promoters

The desired gene expression is determined by several factors where promoter choice plays a significant role. The sequence of core promoter, *cis*- and *trans*-acting elements, 5′-UTR, TATA box, upstream and downstream sequence from the start codon and mRNA secondary structures are key features that determine the protein expression levels [53]. P_{AOX1} is the most extensively employed promoter for protein expression in *Pichia*. The *AOX1* promoter is tightly regulated with a complete repression of transcription in the presence of a carbon source (glucose/glycerol). It exhibits high promoter activity in the presence of an inducer (methanol). However, the yeast promoters are, in general, more complex with higher size (P_{AOX1} 950 bp) than bacterial promoters (<100 bp). Moreover, there is minimal knowledge available related to the molecular mechanisms and *cis*-acting elements of the *AOX1* promoter region, which regulates the protein expression level. In recent years, the underlying mechanism and regulatory elements of the promoter that govern the level of expression are studied. Berg and co-workers used the combinatorial mutagenesis approach employing random mutations to engineer the P_{AOX1} [54].

The combinatorial mutagenesis approach was employed to determine the *cis*-acting elements of P_{AOX1} that govern glucose repression. Two regions of P_{AOX1} were studied, the core region sequence, which spans 15 bp upstream of the presumed TATA box to around 35 bp downstream from the start site of transcription. The library of mutants containing randomly mutated synthetic nucleotides of the core region was cloned individually in a modified pPPE20 vector (which contains Zeocin resistance marker downstream of P_{AOX1}) followed by a transformation in the GS115 strain. A second set of the library was created with mutations in the region 90 bp upstream of the core region of P_{AOX1}. Both these libraries were then screened for enhanced zeocin tolerance compared to the control strain under the presence of glucose as a repressing molecule. A total of five different transformants with enhanced zeocin tolerance were selected. These promoter variants contained 2- to 18-point mutations. Two of the variants having mutations directly upstream of the TATA box had only 2 and 3 mutations, respectively, which indicates the role of these single nucleotide mutations in the glucose repression of P_{AOX1}. Moreover, the other three transformants carried multiple point mutations (11–19) in the core region as well as 90 bp upstream of it. Hence, using combinatorial mutagenesis and selection method, the role of the core region and 90 bp upstream region from the core was identified to be significant in glucose repression of P_{AOX1}. Similarly, the P_{AOX1} variants supporting a higher level of protein expression were screened in the presence of an inducer. The transformants carrying mutations were screened for the presence of increased zeocin tolerance under the P_{AOX1} inducible conditions (methanol). In the final step, three promoter variants were selected, which contained over 5–12-point mutations in the promoter's randomized region, which was also found to be essential for glucose repression. This could be explained by the presence of *cis*-acting elements present in the same region of the promoter and determine the methanol induction and glucose repression properties of P_{AOX1} [54].

Hartner et al. [55] studied the regulatory elements which dictate the expression level of P_{AOX1} using site-directed mutagenesis and overlap extension PCR for promoter library preparation. The study aimed at making deletion mutants and thus identify small regions that function as regulatory elements and classify the transcription factor (TF) that binds and regulates *AOX1* activity. In silico analysis facilitated the identification of putative transcription factor binding sites (TFBS). Using this methodology, the promoter variants with activity ranging from 6 to 160% were generated with the identification of 12 *cis*-acting elements [55]. Mutations within the TFBSs led to a change in promoter strength. Additionally, seven regions in the P_{AOX1} were identified where deletion of either complete putative TFBSs or its small core sequence could decrease the *AOX1* promoter activity by >50%. Further, mutations were also introduced in the *AOX1* promoter, which resulted in altered positioning of *trans*-acting factors.

Ata and co-workers [56] conducted a similar study where they constructed the *GAP* promoter variants. The variants were generated by either the series of deletion or duplication of the transcription factor binding site in the P_{GAP} promoter. The expression level of the reporter gene (GFP) was improved in a range of 0.35–3.1 compared to wild-type promoter variants. Various putative transcriptional factors

were also studied later, which could modulate the activity of P_{GAP}. A GAL-4-like transcription factor was found essential for the improved activity of the *GAP* promoter. An engineered P_{GAP} containing the duplicated region for the binding of GAL-4-like TF and the strain overexpressing this transcription factor showed a 3.1-fold enhancement in GFP expression level [56].

Xuan and co-workers [57] identified a region D located in a position between −638 and −530 as the *cis*-acting element of the *AOX1* promoter using deletion and insertion strategies. Upon insertion of three copies of region D in a strain carrying a deletion of this region in the promoter showed 157% enhancement in expression levels [57]. The 5′ untranslated region has also been reported to be critical for regulating the translational efficiency of the *AOX1* promoter as it contains *cis*-acting elements with positive and negative regulatory functions [58].

Poly (dA:dT) tracts in the yeast genome and *AOX1* promoter play an essential role in transcription regulation and, hence, promoter activity. These tracts also influence the affinity and occupancy of nucleosomes. Yang and colleagues demonstrated that deletion and alteration in the length of Poly (dA:dT) tracts could improve the *AOX1* promoter activity resulting in enhanced production of porcine growth hormone from 0.25 to 3.5. Thus, engineering the Poly (dA:dT) tracts in the promoter region could be an effective strategy to achieve a higher expression level of target proteins [59].

A transcriptional regulator, PpNrg1, has also been recognized as an *AOX1* promoter repressor [60]. The PpNrg1 binding site has a conserved region to those found in the binding site of activator proteins such as Mit1. Thus, the similarity in the binding regions of these antagonistic molecules suggests the competition between the binding of repressors and activators. In another study, Wang and co-workers identified an essential regulator of *AOX1* promoter, namely, methanol-induced transcription factor 1 (Mit1), and defined the role of two other crucial regulatory factors, Prm1 and Mxr1. These factors work in a cascade to regulate the *AOX1* promoter. In the absence of methanol, the Mxr1 localizes to the cytoplasm. In the presence of methanol, the Mxr1 re-localizes to the nucleus and derepresses the P_{AOX1}. De-repression of *AOX1* and the presence of methanol further cause the activation of Prm1, which induces its own expression and also activates the induction of Mit1. Mit1 activation results in robust activation of the *AOX1* promoter. Mit1 also inhibits Prm1 in a feedback loop to prevent its own accumulation [61].

Furthermore, for improved expression of target genes, the strategies have been designed to construct the synthetic promoters that contain both the native promoter and the *cis*-acting element [59]. Vogl et al. proposed the synthesis of a synthetic promoter for *P. pastoris*. For the generation of the synthetic promoter, sequence alignment of a set of promoters was done, leading to the identification of the consensus promoter sequence. Further, the transcription factor binding sites were introduced in the consensus sequence to construct a core promoter. The synthetic core promoters thus generated were fused to the P_{AOX1} upstream region, leading to several promoter variants. These variants exhibited improved AOX1 activity from 10 to 117% compared to the wild-type AOX1, where GFP was used as a reporter gene [62, 63].

A detailed study of yeast core promoter has been done using the high-resolution systematic approach where the 200 bp core promoter sequence from the start codon was studied. The entire region was characterized systematically, where at one time, three adjacent nucleotides in the wild-type promoters were mutated to three AAA or CCC triplets. Using a systemic biology approach, 130 promoter variants were generated with eGFP as a reporter gene. A significant change in the expression of eGFP was seen in the case of nine variants having cytosine-triplet, while triple adenine mutant variants did not show any substantial change in expression level [62]. Thus, the numerous strategies described above could lead to a better understanding of regulatory elements and, therefore, could generate a high strength *AOX1* promoter.

18.3.2 Host Cell Engineering

In order to engineer host strains, three factors need to be considered: (a) complete information regarding the genome; (b) availability of genome engineering tools; (c) existence of sufficient genetic elements such as promoters and different sites for integration, enabling stable gene expression. The genome sequence of *Pichia* has been extensively annotated and available on www.pichiagenome.org. However, there are very few studies that detail the genetics and physiology of the *Pichia* host. The non-homologous end-joining (NHEJ) is a predominant method for double-stranded DNA repair after the transformation of linear DNA. The targeted homologous recombination with knock-out cassette possessing homologous arms is a less efficient and uphill task in the *Pichia* host than *S. cerevisiae*. For specific targeting of genes by knock-in and generation of knock-outs with high efficiency, a DNA helicase II subunit 1, Ku70 homologue gene in *Pichia*, was deleted. Ku70 has been implicated in playing a role in NHEJ. The Ku70 deletion strain (generated by knocking out 215 bp in the *KU70* locus) exhibited improved efficiency (up to 90%) in gene targeting when *ADE1* and *HIS4* were used as integration loci [64]. In another study, the gene associated with NHEJ, the homolog of the *dnl4* gene (DNA ligase IV), was deleted. Further, double mutants were also constructed carrying a deletion in both the *ku70* and *dnl4* genes. The deletion mutant strains were examined to alter the efficiency of targeting genes by gene knock-in and knock-out experiments. The strain carrying deletion in Dnl4 displayed improved chances for homologous recombination in comparison to the control strain and Ku70 deletion strain. Further, the gene targeting efficiency of the *dnl4-ku70* knock-out was more than the single knock-out genes. Hence, these deletion strains with enhanced efficiency for homologous recombination can be employed for further strain improvement as well as for the generation of platforms for functional analysis [65].

The CRISPR/Cas9 gene-editing tool has been successfully employed in *Pichia pastoris* to disrupt specific genes. Weninger and co-workers first established the rapid, marker-less and precise engineering of the *Pichia* genome using CRISPR/Cas9. Using this technique, the gene targeting efficiency could be reached up to 100%. To achieve this aim, the expression of Cas9 protein was optimized [66]. The

gene disruption studies were performed using the optimized CRISPR/Cas9 system involving the knock-out of multiple genomic loci at once and the integration analysis of homologous DNA cassettes. Further, using this strategy in ku70 deletion strains significantly improved single loci and multi-loci integration efficiency. Moreover, a system involving dCas9 protein (inactive endonuclease) has been developed. The CRISPR/dCas9 contains sgRNA that guides the dCas9 for binding to the preferred DNA sequence. Due to inactive nuclease activity, the dCas9 cannot perform DNA-breaking activity. The binding of dCas9 to the favoured sequence prevents the interaction and binding of transcription factors to the DNA, thereby resulting in transcription inhibition. Thus, the CRISPRi technique could identify the regulatory regions in the promoter, which determines the protein expression. For example, the attachment of dCas9 to the sequence present between −468 and −487 regions of P_{AOX1} resulted in the repression of *AOX1* promoter activity [67, 68].

Cai and co-workers [69], employed CRISPR/Cas9 for extensive engineering of yeast. The efficiency of homologous recombination was improved, resulting in enhanced proficiency in effortless gene disruption and integration of multiple genes simultaneously. The study identified 46 sites in the genome for stable integration and expression of target genes with profiling of 18 promoters for gene regulation. A two-factorial regulation strategy was thus established by integrating the characterized promoter and genomic integration sites for fine-tuning the expression of any desired gene. Further, the overexpression of genes involved in homologous recombination, such as *RAD52*, resulted in efficient DNA integration and impeccable gene deletion. Different neutral sites and promoter combinations were used for metabolic engineering of the fatty alcohol biosynthesis pathway, which resulted in a 30-fold improvement in fatty alcohol production (12.6–380 mg/L) [69].

The *Pichia* host was engineered to produce a substantial amount of human membrane proteins, which are the primary target for drugs. The expression of mammalian origin membrane proteins in yeast has always remained a challenging task for researchers due to the differences in the membrane sterols present in the yeast and animal cells. For this purpose, the host's sterol pathway was engineered to produce cholesterol instead of ergosterol as the key sterol. Genes encoding dehydrocholesterol reductase (DHCR7, DHCR24) were integrated into the genome under the constitutive *GAP* promoter. The engineered strain exhibited an increased level of stable human Na, K-ATPase α3β1 production. The cholesterol-producing *Pichia* supported higher expression, activity and stability of Na, K-ATPase α3β1 at the plasma membrane. The engineered strain can be employed to synthesize other membrane proteins [62, 70].

18.3.3 Humanization of Glycosylation Pathway

Efforts have been made to humanize the N-glycosylation of proteins expressed in *Pichia*. The yeasts and humans diverge in the N-glycosylation pathway after generating Man_8GlcNA_2 intermediate. In humans, no further addition of mannose occurs afterwards. In contrast, the α-1,6 mannose residues are added in the

Man_8GlcNA_2 intermediate in yeasts, leading to $Man_9GlcNAc_2$, which further acts as a substrate for hyper-mannosylation. To achieve humanization of N-glycosylation in *Pichia*, the following five steps are mandatory: (a) inhibiting the activity of α-1,6-mannosyltransferase; (b) construction of combinatorial libraries for the correct subcellular localization of enzymes involved in the processing step; (c) employing different sources for catalytic domains; (d) to secure the accessibility of UDP–GlcNAc in the Golgi; (e) efficient and rapid screening method for identification of recombinant glycoproteins with the anticipated structure of oligosaccharide. In the initial step, deletion of the *OCH1* gene involved in the generation of high mannose structures was done to prevent these undesirable molecules, which are difficult to access by α-1,2-mannosidases. Further, in the screening of transformants with the ability to convert the intermediate protein $Man_8GlcNAc_2$ to $Man_5GlcNAc_2$ protein, the OCH1 deletion strain was employed. The deletion strain was transformed with the gene library, which contains the gene encoding synthetic α-1,2-mannosidases required to form $Man_5GlcNAc_2$. The genetic libraries possessing the gene for the catalytic domain of mannosidase enzyme were screened using a reporter gene expression. The deglycosylation was performed to analyse the N-glycans release. The released glycans were characterized by MALDI-TOF mass spectroscopy. *Pichia* was engineered to achieve the next step of humanized N-glycosylation using a similar strategy. For this purpose, the fusion library of leader-GlcNAc transferase-I was constructed [71].

Choi and co-workers [72] employed a similar strategy for engineering the yeast N-glycosylation pathway. They expressed three recombinant genes involved in N-glycosylation: gene coding for the transporter uridine 5′-diphosphate (UDP)-GlcNAc (from *Kluyveromyces lactis*); gene encoding the catalytic domain of mannosidase MnsIA (from mouse) present downstream of the N-terminus of Sec12 (from *S. cerevisiae*) (localization sequence of ER protein); and GnTI, a human GlcNAc transferase, fused with the Mnn9 (leader sequence of Golgi protein from *S. cerevisiae*) in the Och1 deletion strain [72]. Furthermore, for the major production of $GlcNAc_2Man_3GlcNAc_2$ N-glycans, Mannosidase II (ManII) from *Drosophila melanogaster* and GnTII (GlcNAc transferase from rat) genes fused with Mnn2 localization signal of Golgi protein (from *S. cerevisiae*) were overexpressed [73].

Jacobs and co-workers in 2009 engineered the *P. pastoris* strain to produce $Gal_2GlcNAc_2Man_3GlcNAc_2$ N-glycans by transforming the wild-type strain with the GlycoSwitch vectors encoding mannosidases ManI and ManII, GlcNAc transferase GnTI and GnTII and transporter GalT [74]. The O-glycosylation in yeast also differs from the mammalian counterpart. O-Glycans contain the α-1,2-linked mannose with possible phosphorylated mannose or β-1,2-linked mannose at the outer ends. Further, *Pichia* was engineered by integrating the mannosidase gene from *Trichoderma reesei* in the host genome along with the deletion of α-1,2-phosphomannosylation and β-1,2-mannosylation genes resulting in the addition of single mannose O-glycans [75, 76].

18.3.4 Engineering of Secretion Pathway

The α-mating factor from *S. cerevisiae* is the most commonly employed secretion signal for the extracellular secretion of target proteins. In recent years, several novel signal peptide sequences have been recognized. By analysing the genome and secretome of *Pichia pastoris*, various endogenous signal peptides and factors involved in protein folding were screened. Using this methodology, Gas1, Msb2, Fre2 and Dan4 were recognized as the novel signal sequence and Pdi2p, Sil1p and Mpd1 as the folding factors. These putative signal peptides and folding factors were characterized using three different reporter genes (eGFP, β-galactosidase and cephalosporin C acylase). The Msb2 signal peptide improved the production of all the tested genes by 1.5–8.0-fold. Dan4 enhanced the expression of all the three proteins by 172-fold compared to the α-mating factor. The expression of putative folding factors improved cephalosporin C acylase (SECA) expression level where Mpd1 showed maximum effect with 3.1-fold enhancement in SECA extracellular production [77]. Moreover, the genes involved in the cell wall synthesis, such as CWP1, have been disrupted to improve the extracellular secretion of recombinant proteins. The Och1 knock-out strain carrying a deletion of CWP1 resulted in an engineered *Pichia* strain with a high ability to secrete heterologous proteins [78].

In another strategy, the secretory protein of *Komagatella phaffii* extracellular protein X 1 (EPX1) and its truncated variants were tested as an alternative to the *S. cerevisiae* α-mating factor. EPX1 resulted in a high amount of secretory protein expression with authentic N-terminus, thereby decreasing the dependency on the overexpression of KEX2 and STE13 proteases, which are required for native N-terminus generation [62, 79, 80].

18.3.5 Engineering of Terminators

The role of the terminator sequence is crucial for mRNA stability and plays a pivotal role in transcription termination. However, the role of terminators has largely been overlooked, with very few studies covering their relevance in optimizing protein expression. The characterization of terminator sequences (approximately 72 bp) from *P. pastoris* and *S. cerevisiae* and synthetic terminator sequences allowed the fine-tuning of gene expression with an improvement in expression levels up to 17-fold [65]. Additionally, insertion of the *Not*I restriction enzyme sequence in the AOX1 terminator resulted in a 37% enhancement in the expression of eGFP [81, 82].

The size of the *P. pastoris* terminator sequences ranges 174–507 bp, which can be replaced by the synthetic sequence of 35–75 bp or with the terminators from *S. cerevisiae*. Researchers used the terminator sequence of various ribosomal proteins, which are strongly expressed in *Pichia*. The results showed that these alternative terminator sequences from *Pichia* had a similar effect on target gene expression, whereas sequences from *S. cerevisiae* showed slight improvement by a maximum of 50% [76, 83].

18.3.6 Metabolic Engineering of *Pichia*

The heterologous production of recombinant proteins often lowers the host's cellular health, indicating the detrimental effect of foreign gene expression. The division of cellular energy to produce recombinant protein and biomass is not the only explanation for decreased cellular growth. In yeast, this could be attributed to additional bottlenecks such as low copy number of genes and weak promoter activity, resulting in limited transcription efficiency, protein folding, secretion and proteolytic degradation. Furthermore, the metabolic limitations also affect the production level of recombinant proteins.

Various mathematical and computational methods have been devised for monitoring and predicting the effect of recombinant gene expression on the host. The ease in access to the *Pichia* genome sequence has led to the development of genome-scale metabolic models (GEM). Using this model system, the interplay between biomass generation and energy demand upon recombinant protein production can be easily investigated. GEM has been employed to predict the mutations in the metabolic pathway of *Pichia*, which can lead to the hyper-production of target genes. Nocon et al. used a similar approach where manipulations were done in the central metabolic fluxes to overexpress the human superoxide dismutase (hSOD). MOMA (minimization of metabolic adjustment) and FSEOF (flux scanning based on enforced objective function) models were used to predict the possible beneficial targets for gene knock-out and identification of overexpression targets, respectively. The targets for overexpression of specific genes were found in the pentose phosphate pathway and tricarboxylic acid (TCA) cycle, whereas the branch points in the glycolysis pathway were found to be effective targets for gene knock-out. By employing metabolic modelling, five targets were found to enhance the intracellular production of hSOD. Additionally, the observation of flux analysis after the overexpression of hSOD was found to support the prediction data. Thus, the high expression of target genes could be achieved with high accuracy using the metabolic modelling tools [84].

Adaptive laboratory evolution (ALE) is an efficient tool for genetic engineering of *Pichia*. The ALE tool is highly efficient for engineering the genome, especially when the desired phenotype is complex and not well defined genetically. The evolution process by adaptation to a specific environment and reverse engineering has been employed to improve the production level and stress tolerance in many yeast strains [85]. In *Pichia*, the ALE methodology was used to screen for variants with enhanced growth in methanol coupled with increased protein expression [86]. Additionally, high-throughput methods for cloning have been adapted for the rapid engineering of pathways and single expression cassettes. For the *Pichia* system, Golden Gate assembly (GGA), Gibson assembly and restriction site-free cloning (RSFC) strategies were designed [76]. Further, the GGA cloning method has been used for the concomitant gene expression of eight genes using a single vector [83]. The GoldenPiCS kit was designed, allowing the metabolic engineering in different hosts employing the equivalent cloning platform [87]. Gibson assembly was also used for metabolic pathway engineering to characterize 49 promoters and

20 terminators [82]. All these studies have highlighted preventing the use of repetitive sequences while designing vector and pathway engineering [76].

18.3.7 Construction of Methanol-Free AOX1 Promoter System

A methanol-based induction system is the most popular approach for directing the heterologous protein production under the AOX1 promoter. However, the highly volatile nature of methanol, its toxicity and the problem associated with methanol feeding during high cell density cultivation (high oxygen demands and heat generation) demand the development of alternative routes for AOX1 induction. Researchers have developed several engineering strategies to construct a methanol-independent AOX1 system. Wang and co-workers identified the *trans*-acting regulatory elements of the AOX1 promoter and used a combinatorial mutagenesis approach to develop a methanol-free AOX1-inducible system. Three transcription factors (Mig1, Mig2 and Nrg1) were recognized as the transcription repressor of the AOX1 promoter. The double mutants (-mig1, -mig2) and triple mutant (-mig1, -mig2, -nrg1) were generated. These mutants were grown in the presence of glucose and glycerol to study the de-repression of AOX1 in the absence of these repressor molecules. All the mutant strains showed complete repression of AOX1 in 1% glucose or in 1% glucose + 0.5% methanol, indicating that other regulatory molecules strictly regulate the glucose-mediated repression of AOX1. However, higher activity of AOX1 was reported in the presence of 1% glycerol in the case of double and triple mutants. Further, the combination of 1% glycerol and 0.5% methanol resulted in significant induction of the AOX1 promoter. These results showed that deletion of Mig1, Mig2 and Nrg1 could lead to de-repression of AOX1 by glycerol with a tight suppression of AOX1 activity even in low glucose concentration. Additionally, overexpression of Mit1 activator protein under the constitutive GAP promoter in double and triple mutant strains further improved the AOX1 activity in the presence of glycerol or glycerol + methanol. Expression of GFP under the control of AOX1 in the triple mutant strain overexpressing the Mit1 activator showed 77% expression levels compared to the methanol-inducible WT (wild-type) strain. Thus, researchers designed a glucose–glycerol shift induction system based on these results. In the batch phase, a glucose concentration of 40 g/L was used, while in the fed-batch phase, the glucose-based repression of the AOX1 promoter was maintained using 20 g/L glucose. After the complete utilization of glucose, the AOX1 was induced using a methanol-independent glycerol-based feeding system. A 2.46 g/L production of insulin precursor was achieved with a substantial decrease in heat production and oxygen demands [88].

The role of kinases that regulates the activity of AOX1 could be other potential targets for the generation of methanol-free inducible system. Using deletion mutants and colourimetric screening assays, two kinases GUT1 (a glycerol kinase) and DAK (a dihydroxyacetone (DHA) kinase) were recognized as potential targets to modulate the AOX1 repression–de-repression mechanism. Two different strains were developed, one carrying the deletion in gut1 and expressing the *Hansenula polymorpha*

glycerol dehydrogenase enzyme (Δgut1-HpGCY1), and another was having a deletion in the dak encoding gene with DHA as the inducible carbon source (Δdak-DHA). In both these strains, the AOX1 was induced by the non-methanol-based carbon sources (glycerol and DHA). By expressing three different reporter genes in both of these strains, the Δdak-DHA strain was more efficient in terms of AOX1 activity. The DHA-induced AOX1 promoter exhibited higher activity than the constitutive GAP promoter, with 50–60% activity than the AOX1 promoter [89].

Recently, a methanol-free promoter system was introduced in the *Pichia*. The *H. polymorpha* methanol oxidase gene promoter region (pMOX) (533 bp upstream of the MOX gene) was isolated and replaced with the AOX1 promoter region (pAOX1) in the pPINK-HC plasmid, followed by the transformation in *Pichia*. The CMC3 (endoglucanase 3) and EgII (endoglucanase 2) were used as reporter genes to access the activity of pMOX-carrying strain. The pMOX activity was found to be wholly repressed in the presence of xylose and sorbitol, while complete de-repression was obtained in the presence of glucose, glycerol and methanol. The expression profile analysis in protease-deficient strain of *Pichia* showed significant improvement in the production levels of CMC3 and EgII [90]. Further, it was found that the clones harbouring multi-copy genes of the CMC3 and EgII showed higher secretory expression. Thus, the methanol-free pMOX and the protease-deficient strain carrying multiple copies of the target gene could direct the higher expression levels and, hence, be used as an alternative to the pAOX1 methanol-dependent system [90].

Another strategy used the constitutive lipase-producing *Pichia* strain to express two reporter genes (amylase and γ-GGT) where the AOX1 promoter was induced using methyloleate as an inducer. The host was engineered to constitutively express the lipase enzyme under the control of the GAP promoter. After adding methyoleate as an inducer, the lipase secreted in the medium converted the methyloleate into methanol and oleic acid. The methanol produced in the medium further resulted in the induction of AOX1 promoter, while oleic acid was used as the carbon source for biomass production. Thus, a successful Lip + *Pichia* system was developed, which can be induced independently of the methanol [91].

18.3.8 *Pichia* Cell Surface Display

The microbial cell surface display strategy involves the expression of foreign proteins on the surface of a cell by developing a fusion of the foreign protein with a cell wall protein. The fusion protein on the cell surface retains its spatial structure and biological efficacy. The cell surface display approach involves three different components: host cells, carrier protein and passenger protein. The host cell expresses the fusion protein over its cell surface. The carrier proteins comprise outer membrane and cell surface proteins responsible for targeting the desirable protein towards the cell's surface via their signal peptides. The foreign proteins are the passenger that needs to be displayed on the cell surface. The construction of these whole-cell biocatalysts is more beneficial than free enzyme preparation. In cases where

multi-step reactions occur, the surface-displayed enzymes improve the reaction efficacy by bringing the co-factors and enzymes in close association. Moreover, the cell surface eliminates the additional steps of protein expression, harvesting and purification, thereby simplifying the protein synthesis process and making it less expensive. Furthermore, the whole-cell catalysts can be reused, making the process more economically viable. Cells of different microbial origins, including bacteria, yeast and phage, have been used as a host for cell surface display and also implicated in numerous applications such as peptide engineering, vaccines and environmental and medical purposes [92].

Among the yeast, *S. cerevisiae* is the model organism for which this technology was developed. After *S. cerevisiae*, *P. pastoris* is the preferable yeast that has been employed to create a display host. The development of *P. pastoris* cell surface display technique employs a variety of cell wall proteins such as α-agglutinin, a-agglutinin, Sed1p, Flo1p and Tip1p [93]. *P. pastoris* is considered a better alternative for protein display as it prefers an aerobic mode of respiration and can be used to attain high cell density. The surface expression of proteins requires an expression construct consisting of fusion under a strong promoter. Followed by the promoter, a signal peptide is cloned to target the protein to the extracellular medium. The fusion gene comprises a target gene genetically linked to the cell wall anchoring protein [94].

The GPI (glycosylphosphatidylinositol)-anchored proteins are essential for cell viability. These GPI-anchored proteins consist of a GPI attachment site, signal peptide and Ser/Thr-rich sequence responsible for O-linked glycosylation. The GPI-anchored proteins used for cell surface display are commonly isolated from *S. cerevisiae*. However, very few proteins native to *P. pastoris* have been isolated. Therefore, its genome was screened for putative cell wall anchoring GPI-modified proteins. The putative genes were cloned in fusion with the *Candida antarctica* lipase B (CALB) using α-factor as the secretion signal. After confirmation by whole-cell flow cytometry, immunoblotting and digestion with β-1,3 glucanase, the CALB-displaying strains were selected. The putative cell wall proteins native to *P. pastoris* having anchoring capacity were identified [95].

Cell wall proteins native to *S. cerevisiae* are successfully used to target proteins on *Pichia* cell surface. The *S. cerevisiae* Sed1p anchor protein has been used to target lipase of different source organisms such as *Rhizopus oryzae* (ROL) and *Candida antarctica* (CALB) on the *Pichia* cell surface. The ROL-displaying yeast cells were characterized, where it was observed that the surface display of the ROL enhanced the thermal tolerance of the enzyme. Similar results were reported in the case of CALB expressed on *Pichia* cells [96, 97]. The α-agglutinin anchor protein was used to construct the *P. pastoris* cell surface display platform, expressing *Kluyveromyces* yellow enzyme on its surface. A novel surface display system based on α-agglutinin was developed using EGFP (enhanced green fluorescent protein) as the model protein where the expression cassette also comprised Xpress epitope that provided sites for selective cleavage of proteins [98].

PIR (**P**rotein with **I**nternal **R**epeats) are covalently linked proteins that are found in different budding yeast. They have been reported in *S. cerevisiae*, which has

varying numbers of internal repetitive units. These are linked to the β-1,3 glucans via an alkali-labile ester linkage. Two PIR proteins native to *P. pastoris* (Pir1p and Pir2p) were identified, such that the Pir1p protein displayed a pre-pro-type structure at the N-terminal region of the protein. It also consisted of a putative Kex2 cleavage protease site. The Pir protein successfully displayed EGFP on the *Pichia* cell surface, which was further confirmed by fluorescence microscopy [38].

18.4 Applications of *P. pastoris* Expression System

18.4.1 Introduction

Heterologous proteins are a multibillion doller market comprising commercially relevant industrial enzymes and therapeutic proteins. *Pichia pastoris* has emerged as a highly dependent expression host that can express a diverse group of proteins. In the health-care industry, it has been utilized to express several biopharmaceuticals. Its fast growth rate, as well as the absence of endotoxins, bacteriophage contamination and human pathogens, efficient protein folding, glycosylation, methylation and acylation constituting the post-translational modifications, makes it a useful system for recombinant protein expression. On the other hand, enzymes are extremely valuable in the food and feed industry. For animal feed, enzymes such as phytase and xylanase enhance the availability of essential nutrients [99, 100]. Hence, the following section highlights the role of *P. pastoris* expression system as a suitable platform for the production of therapeutics and commercially important enzymes.

18.4.2 Therapeutic Protein Production in *P. pastoris*

18.4.2.1 Growth Factors

Epidermal growth factor (EGF): It is a small mitogenic protein that binds to the epidermal growth factor receptor (EGFR) and stimulates the growth and proliferation of different cell types such as fibroblasts, thyroid and embryonic cells. EGF is a therapeutic protein that helps in the treatment of several pathogenic conditions, thereby highlighting its potential as a major commercial agent. Khan and co-workers demonstrated that the human liver (hepatoma) cell line, *Huh-7*, can be used to extract the partial gene of hEGF whose sequence is 100% homologous to the wild-type hEGF gene. The gene was cloned into the pPICZαA vector and successfully expressed in *P. pastoris* cells. Since EGF has potential glycosylation sites, a high proportion of the protein was expressed in a glycosylated form. From the biological activity assay, both the glycosylated and non-glycosylated forms of the protein were found to be bioactive [101, 102].

Fibroblast growth factor (FGF): It is a group of secreted molecules that signal tyrosine kinases and intracellular non-signalling molecules that act as co-factors for other molecules and voltage-gated sodium channels. It plays a critical role in the initial steps of embryonic development and organogenesis. It is essential for

maintaining, repairing and regenerating adult tissues. It is further involved in cellular processes such as cell proliferation, metabolism, migration and differentiation [103]. The extraction of basic FGF (bFGF) from animal sources is cumbersome and expensive as it yields a low quantity of the product. Therefore, recombinant expression of bFGF was achieved by targeting its secretory expression in *P. pastoris*. The full-length cDNA of the gene obtained from RT-PCR was cloned in the pPICZαA vector, where approximately 150 mg/L of the 18 kDa protein was obtained. The ion-exchange chromatography-purified protein was tested for its biological activity by the NIH/3T3 cell lines. The recombinant bFGF with a purity of 94% stimulated the growth of tested cell lines [104].

The FGF21 plays a crucial role in the protection of the heart and liver as well as in glucose homeostasis. Studies involving the expression of FGF21 in *E. coli* system have proven to be difficult as it yielded low quantities of soluble protein. As a result, Song and co-workers successfully expressed FGF21 gene in *P. pastoris*. The recombinant FGF21 was used to treat fibroblasts exogenously, resulting in the stimulation of cell migration and activation of JNK phosphorylation, thereby regulating wound repair [105].

Human keratinocyte growth factor (KGF): It is a mitogen of the epithelial origin belonging to the fibroblast growth factor (FGF) family. It is the seventh member of the family, also known as FGF7, which, along with its members of the FGF7 subfamily, is expressed in the mesenchymal cells. KGF participates in numerous functions such as cell proliferation, anti-apoptosis, cytoprotection, migration and mitogenicity [106]. It is used to stimulate the healing process of wounds and tissues, which helps in wound healing. The low-level heterologous production of this protein in *E. coli* and its unstable nature have prompted researchers to screen alternative hosts. A truncated form of KGF comprising 140 amino acid residues was cloned into the pPICZαA vector, where its secretory expression was targeted via the α-secretion signal. The recombinant protein (KGF_{140}) was successfully detected in the culture medium, which was further purified via heparin affinity chromatography. The bioactivity assays using KGF_{140} confirmed its proliferative activity towards the NIH3T3 and A549b cell lines. It enhanced the survival rate of irradiated A549 and MCF7 cell lines. In addition, its proliferative effects on the human breast cancer cell line, MCF7, was also studied [107].

Granulocyte colony-stimulating growth factor (G-CSF): G-CSF is a hematopoietic growth factor that is utilized as a therapeutic intervention for neutropenia and leucopenia due to chemotherapy and radiotherapy. Since it mobilizes the progenitor cells for transplantations, it is crucial during bone marrow transplantation and AIDS treatment. Although *E. coli* and mammalian Chinese hamster ovarian (CHO) cell lines are often used for its production, *P. pastoris* expression was beneficial due to its ability to produce biologically active proteins at low cost. Although G-CSF has been expressed in *P. pastoris* with the yield varying from 2 to 250 mg/L, the majority of the product has been obtained in an aggregated form or requires surfactants. Maity and co-workers cloned and expressed the G-CSF gene under *GAP* and *AOX1* promoter regulation in the *P. pastoris* SMD1168 strain. The expression under the *GAP* promoter resulted in cellular toxicity, while the *AOX1*

promoter yielded approximately 4 mg/L of protein. High-level production in a 5-L chemically defined medium resulted in a productivity of around 0.7 mg/L/h, which was 4-fold higher than the shake flask yields [108]. It has been observed that glycosylation is not a necessary PTM for the functional aspects of G-CSF. However, glycosylation is known to make protein more stable and resistant to proteolytic degradation. Thus, compared with the non-glycosylated form of G-CSF in *E. coli*, the glycosylated form in *P. pastoris* is preferred. Moreover, in the case of CHO cells, its production may be hampered by viral contamination. A protein fusion technology was proposed to produce therapeutic protein, where the 647 bp fragment of human serum albumin's (HSA) domain III was fused with the 547 bp fragment of G-CSF. The secretion efficiency of the fusion protein was tested with the α-factor secretion signal regulated by the *AOX1* promoter, where the protein concentration varied between 110 and 380 mg/L. The biological efficacy of the fusion protein was tested by treating the THP-1 cell line with the recombinant fusion protein and a positive control G-CSF (Filgrastim), where cell proliferation was observed in the case of treated cells in comparison to the untreated cells [109]. Further, enhancement in the production of a codon-optimized copy of G-CSF was obtained by carrying out mutations in the pro-region of the α-secretion signal leading to G-CSF yields of 39.4 ± 1.4 mg/L [110].

Human granulocyte-macrophage colony-stimulating factor (hGM-CSF): hGM-CSF is a hematopoietic growth factor responsible for differentiating and proliferating granulocyte and macrophage in the bone marrow. It is a therapeutically relevant molecule used to treat cancers such as myeloid leukaemia. Since it stimulates granulocyte and macrophage colony proliferation, it finds applications for immuno-compromised patients and individuals suffering from neutropenia and aplastic anaemia. The heterologous expression of hGM-CSF is hampered by its toxic nature. The constitutive expression of this protein has been targeted in *P. pastoris*, yielding 90 mg/L of secreted protein, whereas a constitutive-inducible combined expression led to the secretion of 180 mg/L of the protein. The fed-batch cultivation on a complex feed resulted in product titers of 250 mg/L. On the other hand, continuous cultivation resulted in 82 mg/L of recombinant protein in a concentrated complex medium with a specific product formation rate of 0.5 mg/g/h at 0.2 h^{-1} dilution rate [111–113].

Platelet-derived growth factors (PDGFs): These molecules participate in several phenomena, such as chemotactic recruitment and cell proliferation, where they serve as a mitogen for several kinds of cells. PDGF-AA participates in embryonic development and postnatal migratory responses. It was successfully expressed in *Pichia* and purified using ion-exchange chromatography (20 mg/L) in glycosylated form with a purity percentage of 95%. Further, its biological efficacy was analysed using NIH/3T3 cell proliferation assay, where 0.1 ng/mL of rhPDGF-AA was sufficient to stimulate the cellular growth [114].

Vascular endothelial growth factor (VEGF): It is a mediator of tumour-induced angiogenesis. VEGF ligand has found clinical applications in the production of anti-tumour drugs. There are different isoforms of VEGF in humans. VEGF165b is a native anti-angiogenic isoform associated with cancer, systemic sclerosis and

ischemic retinopathy. The fusion of human serum albumin (HSA) helps in improving the half-life of the protein; therefore, HSA–VEGF165b fusion protein was successfully expressed under the *AOX1* promoter in *P. pastoris* to a level of 275 mg/L. The fusion protein showed a 20-fold higher serum half-life over the recombinant VEGF165b in mice experimental model [115]. A similar fusion strategy was effective when recombinant VEGF-A_{165} was genetically linked with the N-terminus of *Trichoderma reesei* Hydrophobin II (HFBII) and expressed in *P. pastoris* [116].

Hepatocyte growth factor (HGF): It is also known as scatter factor and a crucial mitogen that helps endothelial cells, fibroblasts, epithelial cells and melanocytes to proliferate. It also inhibits tumour-based cell lines. A recombinant variant of HGF was expressed in the culture supernatant of *P. pastoris* to a level of around 8.0 g/L at a cellular biomass of 135 g (DCW)/L using 5-L fermenter [117].

18.4.2.2 Cytokines

Interleukin 1β (IL-1β), a pro-inflammatory cytokine, plays a substantial role in various pathological and physiological responses. Recombinant expression of human IL-1β has been done in the *P. pastoris*. Li and co-workers cloned the cDNA encoding hIL-1β into pPICZαA vector, followed by X-33, GS115 and SMD1168 *Pichia* strains transformation. From shake flask results, the highest production was attained in the X-33 *Pichia* cells. Bioprocess optimization improved the product yield, where 250 mg/L of hIL-1β was produced. Using the two-phase aqueous extraction and chromatography for purification, the hIL-1β protein was obtained with 95% and 85% purity and recovery yield, respectively [118].

Interleukin 2 (IL-2) is a pharmacologically essential cytokine produced by T-lymphocytes. It possesses high therapeutic relevance with a role in renal cell carcinoma and melanoma therapy. Several studies report the production of recombinant IL-2 in *P. pastoris*. The recombinant human IL-2 (hIL-2) has been effectively produced in *P. pastoris* GS115. The hIL-2 was secreted in the culture medium with final purity of 98% at a concentration of 0.45 mg/mL. The recombinant hIL-2 thus produced was biologically active in enhancing the proliferation of CTLL-2 cells and $CD4^+/CD8^+$ ratio [119]. Moreover, IL-2 has been produced as a fusion protein in *Pichia*. Lei and co-workers reported the production of hIL-2 in fusion with human serum albumin (hIL-2-HSA). The purity of the hIL-2-HSA was found to be 95%. Biological activity analysis of fusion protein showed the high activity of hIL-2 in enhancing the proliferation of CTLL-2 cells. Additionally, the *Pichia*-produced hIL-2-HSA exhibited an improved half-life in plasma in the BALB/c mouse model [120].

Interleukin 3 (IL-3) cytokine is a pluripotent hematopoietic colony-stimulating factor derived from the T-cells. This cytokine is obligatory for the proliferation, differentiation and survival of the hematopoietic progenitor cells. It possesses high therapeutic potential in treating haematological malignancies, immunodeficiency disorders, cytopenia, bone marrow transplantation and different cancers. Thus, various efforts have been made for the high-level expression of the hIL-3 gene. Dagar et al. expressed the codon-optimized gene of hIL-3 in *P. pastoris* X-33 and

GS115 cells under the regulation of methanol-inducible AOX1 system and constitutive GAP promoter. Optimization of the best host-vector combination resulted in maximum expression of hIL-3 at a level of 145 mg/L under the tightly regulated AOX1 promoter in GS115 host cells. To improve the volumetric product concentration of hIL-3, bioprocess studies were done in batch and fed-batch modes. The His-tagged hIL-3 was produced at a maximum concentration of 382 and 388 mg/L using batch and fed-batch cultivation strategies, respectively. Non-tagged hIL-3 production in batch fermentation was at a level of 475 mg/L, which was 3.28 folds higher than shake flask results [121]. Furthermore, bioprocess optimization and multi-copy integration of hIL3 resulted in 2.23 g/L of hIL3 in batch fermentation [51].

Interleukin 4 (IL-4), a pleiotropic cytokine, is produced by basophils, neutrophils, NK cells and T-helper 2 (Th2) cells. IL-4 activity results in several pathological and physiological conditions such as hypersensitivity, asthma, tumour immunology and bone loss. The hIL-4 has been shown to contain two potential N-glycosylation sites (N38 and N105). The production of hIL-4 in *Pichia* resulted in a 15.0 mg/L product yield. To study the effect of glycosylation on *Pichia*-produced hIL-4, two mutants were constructed (N38A and N105L) using site-directed mutagenesis. From the results, it was found that hIL-4 get glycosylated only at the N38 position. Further, it was found that glycosylated hIL-4 had lower activity compared to the non-glycosylated form [122].

Interleukin 5 (IL-5) is involved in the activation and differentiation of eosinophils and is produced by type 2 T-helper lymphocytes (Th2). Yan and co-workers reported the production of chicken interleukin-5 (ChIL-5) in *Pichia* X-33 host cells [123].

Interleukin 6 (IL-6), a multifunctional cytokine, also known as 'myokine' (produced by muscle), plays essential biological functions with an influential role in the central nervous system, immune system and bone marrow [124]. Further, IL-6 has been implicated in regulating haematopoiesis, acute-phase responses, immune responses, cell survival, proliferation and apoptosis. IL-6 has also been shown to activate Janus kinases (JAK) and effectors molecule STAT1 and STAT3 after interacting with the receptor [125, 126]. The heterologous expression of hIL-6 was carried out in *Pichia pastoris*. The gene coding for human hIL-6 was cloned in the pPICZα-A vector. Expression studies in *Pichia* X-33 at shake flask level resulted in 280 mg/L of hIL-6. The hIL-6 was further recovered by PEG-8000 precipitation, followed by purification using anion exchanger DEAE and gel filtration (Sephadex G-75), resulting in a 95% pure product. The functional activity of hIL-6 was confirmed by MTT assay [126].

Interleukin 7 (IL-7) is involved in the development and survival of myeloid and lymphoid cells. The IL-7 has several clinical applications to treat various conditions such as infectious diseases, cancer immunotherapy, bone marrow and organ transplantation. Thus, to target the higher production of recombinant hIL-7, its expression was studied in *P. pastoris*. Using large-scale production strategies, 55 mg/L of hIL-7 was produced in the culture supernatant. Further, purification using SP Sepharose chromatography resulted in a 95% pure product with a total recovery yield of 63%

(35 mg/L). Biological activity analysis showed that 10 pg/mL of hIL-7 had similar bioactivity to commercially produced IL-7 [127].

Interleukin 8 (IL-8), a chemokine secreted by monocytes and endothelial cells, functions as a mediator in angiogenesis. The production of active hIL-8 was targeted in *Pichia* to recover the hIL-8 protein in a soluble fraction. The cDNA of hIL-8 (237 bp) was cloned in the pPICZαA vector, followed by the transformation in *Pichia* X-33 cells. Large-scale fermentation studies for hyper-production of hIL-8 were conducted in a 5 L bioreactor, where 225 mg/L of soluble protein was secreted in the culture supernatant. The protein was precipitated using the ammonium sulphate precipitation method, followed by purification using ion-exchange chromatography (CM Sepharose FF column) with a purity of 95%, yielding 30 mg/L of the final product. The hIL-8 displayed high biological activity to induce the migration of mouse neutrophil cells at 0.25 ng/mL concentration [128].

Interleukin 10 (IL-10) is a cytokine with anti-inflammatory properties. The heterologous expression of hIL-10 was done in the *P. pastoris* GS115 strain. It was reported that hIL-10 production under AOX1 promoter with prolonged methanol induction at a lower cultivation temperature of 20 °C resulted in a high expression yield of hIL-10 compared to 30 °C due to lower ER stress [129].

Interleukin 11 (IL-11), a pleiotropic stromal-cell derived cytokine, plays an essential role in megakaryocytopoiesis, thereby increasing platelet production. IL-11 also stimulates the osteoclasts activation, resulting in inhibition of apoptosis, proliferation of epithelial cells and inhibiting the production of macrophage mediators. Additionally, IL-11 also plays a role in erythrocytopoiesis, neuronal development, production of acute-phase proteins and inhibition of adipocyte differentiation and lipase activity. The hIL-11 is a 177 amino acid non-glycosylated peptide. The heterologous expression of hIL-11 was done using the PichiaPink expression system, resulting in its production of 0.31 g/L. The high volumetric concentration of the IL-11 was targeted by conducting fermentation studies, followed by precipitation of the protein secreted in the supernatant and recovery using a two-step of purification (cation-exchange chromatography and hydrophobic interaction chromatography) after the refolding of protein [130].

Interleukin 15 (IL-15) is a pleiotropic cytokine that induces the differentiation and proliferation of NK cells, B cells and T cells. It plays an essential role in the production of $CD8^+$ T memory cells. The recombinant hIL-15 production in *Pichia* was done by cloning the hIL-15 gene in pPICZαA with a C-terminal 6× His tag and c-Myc tag. It was expressed at a maximum of 75 mg/L in *Pichia* X-33 host cells. The secreted protein was further purified using affinity chromatography and DEAE anion exchange at the second step, resulting in 95% purity of hIL-15. The *Pichia*-produced hIL-15 was found to be biologically active in increasing the cellular proliferation of NK cells and CTLL-2 cells [131]. The production of bovine IL-15 has also been reported in *Pichia* [132].

Interleukin 17 (IL-17) is produced by stimulated $CD4^+$ T-cells. It stimulates the secretion of G-CSF, IL-6 and IL-8. The properties of *Pichia*-produced recombinant hIL-17 were similar to the mammalian counterpart. The hIL-17 showed the authentic N-terminus with similar glycosylation patterns to that of human-produced IL-17.

Using two-step chromatography techniques and cation-exchange and gel-filtration chromatography, a 3.5 mg of hIL-17 was produced [133]. Further, Zhou and co-workers also produced the glycosylated monomeric form of hIL-17, which could stimulate 3T3 mouse fibroblast cells to secrete IL-6 [134].

Interleukin 18 (IL-18) belongs to the IL-1 cytokine family. It is involved in several autoimmune diseases, metabolic syndrome, psoriasis, myocardial function, acute kidney injury, macrophage activation syndrome and inflammatory bowel syndrome. The production of mature chicken IL-18 was targeted in *Pichia* using codon optimization of three codons according to *Pichia* codon preference [135].

Interleukin 21 (IL-21) is a cytokine majorly produced by $CD4^+$ T-cells. The cDNA of hIL-21 was cloned in the pPICZα-A vector and expressed in the GS115 *Pichia* strain. The hIL-21 was produced at a maximum level of 229.28 mg/L after methanol induction. In biological assays, the hIL-21 promoted human lymphocytes proliferation [136].

Interleukin 22 (IL-22), an IL-10 family member, plays an essential role in preventing epithelial cell damage and promoting antimicrobial defence. The therapeutic role of IL-22 in autoimmune diseases demands its high production. IL-22 production has been targeted in *Pichia* via gene cloning in the pPICZαA vector. Purification of hIL-22 using SP Sepharose FF column resulted in the efficient recovery of protein with 90% purity with a final product yield of 100 mg/L. The biological activity analysis using HepG2 cells showed high activity of hIL-22 that was comparable to commercial products [137].

Interleukin 23 (IL-23) induces the $CD4^+$ T cell differentiation and is a potential target for treating chronic immune-inflammatory disorders. The mice IL-23 (mIL-23) was expressed in *Pichia* X-33 cells under the control of strong methanol-inducible AOX1 promoter. The mIL-23 was able to induce the proliferation of PBMC (peripheral blood mononuclear cells) [138].

Interleukin 25 (IL-25) is a member of the IL-17 family that participates in the induction of IL-13, IL-4 and IL-5 expression and stimulates Type 2 T-helper cell response in different organs. The high-quality secretory expression of hIL-25 was targeted in *P. pastoris* X-33 host. According to *Pichia* preferential codons, the gene was codon-optimized and cloned in the pPICZαA vector. Expression studies were conducted at bench scale fermenter level followed by protein purification using culture supernatant. Functional analysis of hIL-25 revealed the bioactive nature of the protein with its ability to induce G-CSF production after binding to its receptor IL-17BR in the in vitro studies. Further, the *Pichia*-produced hIL-25 could induce the apoptosis process in the HBL-100 and MDA-MB-231 breast cancer cells [139].

Interferons (IFNs) are the first line of defence produced by host cells against infectious agents and tumour progression. They possess immuno-modulating and anti-proliferative functions with the ability to stimulate cell growth, metabolism and differentiation. IFNs have been categorized into type I and type II IFNs. Type I includes IFN-α, -β, -κ and -ω, while type II has only one IFN-γ. IFN-α is a multigene family consisting of 13 different proteins [140, 141]. The following section details the expression status of various IFNs in the *Pichia* system.

Interferon-α (IFN-α) is known to possess antiviral properties, with enormous potential to be used as a vaccine adjuvant. Porcine IFN-α has been implicated as a therapeutic agent in treating foot and mouth disease and decreasing the occurrence of respiratory and reproductive syndromes. High-level secretory expression of porcine IFN-α was achieved using a combination of secretory signal, codon-optimization, multi-copy gene integration and with the help of the AOX1 promoter. The codon-optimized IFN-α protein was secreted using an engineered α-mating factor (MF4I) to 17.0 mg/mL in six-copy *Pichia* integrant. Its biological activity was tested on Madin-Darby bovine kidney (MDBK) cells where the antiviral activity was reported as $2.8 \pm 0.9 \times 10^9$ IU/mL [142]. Tu and colleagues reported the expression of biologically active codon-optimized IFN-α protein to 200 μg/mL at shake-flask with strong antiviral activity in different cell lines: 1×10^7 AU/mg in MDBK cells and 1.5×10^6 AU/mg in IBRS-2 cells [143]. Further, high production of porcine IFN-α has been achieved using methanol/sorbitol co-feeding or by inducing the cells at a lower temperature [144].

Interferon-α1 (IFN-α1/IFN-αD), a type I IFN, has been used to treat several viral diseases. It has various clinical applications, such as hepatic fibrosis (caused by hepatitis B virus), bovine respiratory disease (in calves), and keratitis (caused by herpes simplex virus). Recombinant hIFN-α1 production in *Pichia* under the AOX1 promoter resulted in a high product concentration of 0.7 mg/20 mL culture along with highly active hIFN-α1 molecules [145]. Further, the stability of recombinant hIFN-α1 was enhanced by constructing a fusion of human serum albumin and hIFN-α1. The fusion protein was expressed using the HSA signal peptide and purified by ion-exchange chromatography (Blue Sepharose and CM Sepharose FF column) with 95% purity. The antiviral activity of the HSA–hIFN-α1 fusion protein was estimated using a cytopathic effect assay at 1.63×10^5 IU/mg. In in vivo assays, an improved biological activity was reported with enhanced stability and retention of HSA–hIFN-α1 [146]. Moreover, a bovine hIFN-α1 has been produced in *Pichia*, which displayed similar characteristic properties to IFN-α [147].

Interferon-alpha 2b (IFN-α2b) is a type I IFN, with several clinical applications in treating AIDS-related Kaposi's sarcoma, melanoma, hairy cell leukaemia and hepatitis B and C chronic conditions. The lymphocytes secrete it as a primary defence upon virus infection. Thus, due to the therapeutic relevance of IFN-α2b, several studies have been conducted to target its higher heterologous expression. Ghosalkar and co-workers produced it in *Pichia* to a level of 200 mg/L by optimizing secretory signal sequences [140]. In another study, the IFN-α2b gene was expressed to a level of 1.5 g/L in an optimized basal salt medium. Further, the use of 10% DMSO in the fermentation broth resulted in the production of a correctly processed isoform of IFN-α2b, with a drastic reduction in the 20 kDa contaminant isoform of IFN-α2b [141]. Additionally, an HSA-fused IFN-α2b (HSA–IFN-α2b) has been produced with long-lasting serum retention and high biological activity [148]. Ayed and co-workers designed the fed-batch fermentation and a two-step purification strategy for optimal production and purification of IFN-α2b. To prevent the proteolytic degradation during fed-batch run, 0.1% casamino acid was included in the culture medium along with 10 mM EDTA, with further changing the

cultivation medium with a fresh medium after the end of the glycerol-based fed-batch phase. The optimization of fermentation and purification resulted in 180 mg/L purified IFN-α2b [149]. In a recent study, a glycoengineered *Pichia* strain GlycoSwitch® SuperMan5 was used to produce glycosylated IFN-α2b with $(Man)_5(GlcNAc)_2$ pattern. The study used artificial intelligence-based optimization of culture medium for efficient production of bioactive IFN-α2b. Under the optimized conditions, a maximum of 436 mg/L IFN-α2b was produced in the bioreactor with high biological activity, as evident from the ability of IFN-α2b to inhibit the growth of breast cancer cells in antiproliferative assays [150].

Interferon-β (IFN-β), a cytokine with therapeutic properties, displays antiviral and antiproliferative activity, similar to that of IFN-α. Heterologous production of porcine IFN-β has been reported in *Pichia*. The amino acid at positions 3, 7 and 164 was codon-optimized following *Pichia* preference and produced at a 60 mg/mL concentration and showed antiviral activity of 2.5×10^5 U/mL. The recombinant porcine IFN-β successfully inhibited the replication of Pseudorabies virus in the MDBK cell line [151].

Interferon-β1 (IFN-β1) is an immunomodulatory, antiviral, antiproliferative molecule consisting of 166 amino acids with implications for treating several diseases. The expression of the human IFN-β1 gene in *P. pastoris* GS115 under the AOX1 promoter resulted in secretory production of bioactive hIFN-β1 having activity of $2–3 \times 10^7$ IU/mg in cytopathic in vitro assays [152].

Interferon-γ (IFN-γ), a class II cytokine, is produced by NK cells, NKT cells and cytotoxic T1 lymphocytes (CD4 and CD8). It is a molecule of therapeutic relevance with a role in several biological functions. These include increasing the lysosomal activity of macrophages and antigen representation, antiparasitic and antiviral activity and effects on cell proliferation and apoptosis. Further, IFN-γ has been approved by the FDA to treat severe malignant osteoporosis and chronic granulomatous. The hyper-production of hIFN-γ was achieved to a level of 200 μg/L in the GS115 *Pichia* strain. Further, by co-expression of the gene encoding for chaperon (protein disulphide isomerase, PDI), a 2.67-fold enhancement in hIFN-γ production was seen. Codon-optimization of hIFN-γ gene further improved the product titers by 9-fold to 1.8 mg/L. Additionally, the optimization of process parameters like pH, temperature, inoculum size, agitation rate and methanol concentration resulted in a 12.5-fold improvement in hIFN-γ production with a final yield of 2.5 mg/L [153]. Medium optimization using statistical algorithms (Plackett-Burman, Box-Behnken and artificial neural network-linked genetic algorithm) led to the optimal production of hIFN-γ at a level of 30.99 mg/L. The use of Luedeking-Piret (L-P) model resulted in 40 mg/L production of hIFN-γ [154]. Further, metabolic engineering of PPP (pentose phosphate pathway) of *Pichia* resulted in enhanced production of hIFN-γ. A 1.9-fold improvement in product concentration was obtained after 6-phosphogluconate dehydrogenase (GND2) overexpression. Whereas the overexpression and synergistic effect of D-ribulose-5-phosphate-3-epimerase (RPE1) and 6-phosphogluconolactonase (SOL3) showed a 2.56-fold improvement in the hIFN-γ concentration [155].

Interferon-γ3 (IFN-γ3), also known as IL28B, is a biotherapeutic molecule. The production of bovine IFN-λ3 has been demonstrated in *Pichia*. The protein was successfully secreted into the culture medium and purified using SP Sepharose ion-exchange chromatography. The bovine IFN-λ3 was produced as an active molecule as it induced the interferon-stimulated genes in bovine PBMCs [156].

Interferon-ω (IFN-ω) possesses potent antiviral, antiproliferative and immunomodulatory activities. In a recent study, the production of feline IFN-ω has been reported using the *Pichia* expression system. The codon-optimized gene was expressed in the *Pichia* GS115 strain, followed by modification using polyethylene glycol for in vivo and in vitro activity assays. The *Pichia*-produced feline IFN-ω showed effective antiviral activity with the ability to treat cat viral diseases [157].

Interferon-ω1 (IFN-ω1) is a type I interferon, which possesses antiviral, anti-tumour and antiproliferation activity. The bovine IFN-ω1 has been produced in *Pichia pastoris*. Self-aggregation of IFN-ω1 was observed in *Pichia*-produced recombinant product. IFN-ω1 was solubilized in urea, followed by desalting and purification using Q Sepharose Fast Flow. The protein was recovered with 80% purity, with a 300 mg/L yield from the fermentation medium and a specific activity of 10^8 IU/mg. The *Pichia*-produced bovine IFN-ω1 exhibited high antiviral activity and showed antiluteolytic activity in cattle [158].

Tumour necrosis factor-alpha (TNF-α): TNF-α is a pleiotropic cytokine that participates in inflammatory reactions and anti-inflammatory processes like tissue repair during the clearing of infections. At low levels of expression, it contributes to tumour development. However, upon exposure to an inflammatory condition, TNF-α, along with IL-1 and chemokines, is expressed by activated macrophages and myeloid cells. It leads to the attraction and activation of neutrophils and monocytes to the site of tumour formation. Its anti-tumour effects are mediated by vascular and necrosis destruction [159]. Due to its importance in the clinical field, the human TNF-α gene was amplified from the phytohemagglutinin-stimulated peripheral mononuclear blood cells and expressed in *Pichia pastoris*. After Ni-NTA purification, an MTT assay was performed to check the biological activity of the recombinant TNF-α, where it effectively diminished the viability of HEP2 cells [160].

18.4.2.3 Subunit Vaccine

The recombinant subunit vaccines contain one of the components which elicit the antigenic response and an effective adjuvant. The subunit vaccine encompasses components produced in the microbial host system. Additionally, they have provided several advantages over live-attenuated or inactivated vaccines, such as better safety. They contain only synthetic or recombinant peptides without including infectious agents (viruses) in the vaccine preparation. For the construction of subunit vaccines, the viral antigens that induce the immune response must be known. The *P. pastoris* system has been successfully used for the large-scale production of viral antigens and, hence, the development of subunit vaccines [161]. The various proteins expressed in *Pichia* for vaccine development are detailed below. The production of viral antigens of Epstein-Barr virus (EBV) has been reported in *Pichia*.

EBNA1 (EBV nuclear antigen 1) is recognized as one of the potential antigens for vaccine development. The production of a truncated form of EBNA1 (390–641 aa) at a concentration of 210.53 mg/L was reported in *Pichia*, with the ability to induce a robust immune response in mice models [162].

In another study, the Dengue virus (DENV) Envelope domain-III (ED III) antigen was successfully developed in the *Pichia* system with high secretory expression using casamino acid supplementation. The recombinant ED III elicited an immune response with substantial induction of neutralizing antibodies [163]. Recently, Shukla and co-workers demonstrated the development of multivalent virus-like proteins (VLPs) to treat two DENV serotypes using the *Pichia* expression system. The E ectodomains of DENV-1 and DENV-2 were co-expressed by cloning the E1 and E2 genes in a head-to-tail manner under the AOX1 promoter. The E1 and E2 proteins were co-expressed and purified, and then co-assembled into VLPs, presenting the EDIII of both E1 and E2 proteins. These bivalent VLPs displayed high immunogenicity, resulting in antibody production, recognizing different DENV serotypes. The generation of virus-neutralizing antibodies proved the efficacy of bivalent EDIII-specific VLPs in BALB/c mice [164].

The gene encoding for Neuraminidase (NA) antigen of H5N1 avian influenza virus was cloned in *P. pastoris*. The recombinant NA was produced at a concentration of 2 mg/L and showed a significant immune response in mice models [161, 165]. Further, the surface expression of Haemagglutinin (HA) antigen protein was carried out in *Pichia*. It was expressed as a fusion to the C-terminus of GPI-anchored α-agglutinin (from *S. cerevisiae*) cell wall protein. The surface-displaying HA yeast showed the production of virus-neutralizing antibodies [166]. Additionally, the production of HA antigen of swine influenza A virus (H1N1) for subunit vaccine development in *Pichia* has been reported. The bioprocess optimization strategy using the *Pichia* fed-batch culture resulted in 1.62 g/L of recombinant protein [167].

The Envelope protein (E) of the Japanese encephalitis virus (JEV) was produced successfully in *Pichia* in glycosylated form with 95% purity. The *Pichia*-produced E protein showed potential results in treating JEV infection by effectively stimulating the immune response [168]. Heterologous expression of Enterovirus 71 (EV71) (responsible for hand, foot and mouth disease) capsid protein VP1 in *Pichia* has shown to be a potential candidate for vaccine development. Expression studies exhibited the successful secretory production of VP1 at a level of ~500 mg/L. Vaccination of mice with the recombinant VP1 resulted in an effective immune response [161, 169, 170]. The production of virus-like particles (VLPs) of Hepatitis B virus surface antigen (HBsAg) was reported in *P. pastoris*. A 99% pure HBsAg VLPs was obtained, which elicited a significant cellular immune response and high titers of HBsAg-specific IgG. Thus, the VLPs produced in *Pichia* are promising candidates for vaccine development [171].

In a recent study, the receptor-binding domain protein of SARS-CoV-2 was expressed in *Pichia*. A total of 30–40 mg/L of protein was produced at the fermenter level. The purified protein was tested for its ability to bind the angiotensin-converting enzyme (ACE2) receptor. The 50 μg of recombinant protein was further

formulated with alum and used in immunogenicity assays, where a high-level RBD-binding antibodies were detected in mice. In in vitro assays, the mouse sera showed inhibition of binding between the receptor-binding domain protein and the ACE2 receptor and neutralized virus infection in Vero E6 cells. Thus, the *Pichia*-expressed receptor-binding domain protein is a suitable candidate for vaccine development against SARS-CoV-2 [172].

The yeast cell surface display technology was employed for the surface expression of ZIKV proteins for vaccine development. Cell wall components of yeast are known to have immunostimulatory properties. The expression cassette was designed to favour the anchoring of the Envelope and NS1 protein epitopes. The EnvNS1 epitopes successfully induced the activation of immunological cells, increasing cytokine levels (TNF-α, IL-6 and IL-10) and enhancing lymphocyte numbers (CD4+, CD8+ and CD16+). The effective immune response generated by the epitopes suggests active vaccine development against ZIKV [173].

Human papillomavirus (HPV) is the causal agent of the most commonly prevalent sexually transmitted cervical cancer. The commercially available HPV vaccine is expensive to develop, and hence alternatives are needed to produce cost-effective HPV vaccines. The HPV16L1 and HPV18L1 were individually cloned in the pPICZ vector, followed by the transformation in *Pichia* KM71 host cells. The purified HPV proteins were formulated with aluminium phosphate as an adjuvant with final HPV16L1 and HPV18L1 concentrations of ~40 and ~ 20 μg/mL, respectively. The *Pichia*-produced HPV vaccines (both HPV16L1 and HPV18L1) were able to induce the production of HPV-specific neutralizing antibodies [174].

Hand, foot and mouth disease (HFMD) is caused by Coxsackievirus A16 (CA16) and affects millions of children in the Asia-Pacific region. Zhang and co-workers proposed the development of VLPs of CA16 in *P. pastoris*. The P1 and 3CD proteins of CA16 were co-expressed in *Pichia* and found to assemble into virus-like particles in high titers. Further, CA16-VLPs induced high antibody production with neutralization effects on CA16. Thus, the CA16-VLPs produced in *Pichia* are potential candidates for vaccine manufacturing against CA16 on an industrial scale [175].

18.4.2.4 Other Therapeutics

Erythropoietin (EPO): EPO is a glycoprotein that possesses pleiotropic functions. It is responsible for regulating the production of red blood cells. Produced by the kidney cells in adults, it plays several functions, including erythroid progenitor cell differentiation, cellular proliferation and inhibition of apoptosis. The deficiency of this hormone results in anaemia, so the FDA has approved its use in anaemic patients who have cancer, kidney failure and chronic inflammatory conditions. Its production has been targeted in CHO cells; however, the efficacy and expenses associated with the mammalian system have resulted in the exploration of alternative hosts. *P. pastoris* has been successfully used for EPO production, where the cDNA was fused with the target site of factor Xa protease. The characterization of glycosylation pattern revealed that all the N-linked sites were glycosylated by the $Man_{17}(GlcNAc)_2$ residues. Strategies to improve the half-life of the protein were

developed by carrying out PEGylation. Glycoengineered *P. pastoris* cells produced recombinant EPO with terminal sialic acid residues and bi-antennary N-glycans structure. The modified glycosylation pattern of recombinant EPO demonstrated better in vitro efficacy over the commercial product [176, 177].

Thrombopoietin (TPO): *TPO* is a potent cytokine that helps megakaryocytes to differentiate and proliferate. It also participates in platelet production. TPO acts synergistically with other hematopoietic molecules. Since IL-6 has the potential to expand hematopoietic stem and progenitor cells, it is expected that IL-6 can work synergistically with TPO for megakaryopoiesis and thrombopoiesis. Hence, a study was carried out to produce IL-6-linker-TPO fusion protein (ZH646) in *P. pastoris*. With a yield of 30 mg/L, the purified fusion protein showed biological activity by exhibiting thrombopoietic effect in mice to increase the platelet counts [178]. A TPO mimetic peptide (TMP) has been recognized that binds and causes the activation of c-Mpl, whose natural ligand is TPO. Since it showed similar biological efficacy to TPO, TMP was used to produce a recombinant analog of TPO. HSA was fused with a tandem repeat of TMP (TMP-TMP-HSA and HSA-TMP-TMP) and transformed into PichiaPink cells. The HSA-TMP-TMP peptide was successfully expressed with a yield of 0.4 g/L in a 20-L fermenter which, when administered subcutaneously to the healthy mice, prompted platelet production [179].

Adiponectin: It is an adipokine secreted by adipocytes involved in lipid and glucose metabolism. It comprises an N- and C-terminal region consisting of a collagen-like sequence and globular domain, respectively. A decline in the blood plasma levels of adiponectin is observed in patients suffering from obesity, which marks an onset of diabetes mellitus. Hence, as a clinical application, adiponectin treatment can help reduce body weight and improve insulin resistance. The globular domain of adiponectin has been expressed in *P. pastoris*, where the production of 1.2 g of protein was detected in the culture supernatant. Further, its biological activity was investigated by recording the clearance of free fatty acids (FFAs) in mice from its plasma containing a high concentration of FFAs. The clearance was comparably faster than the control mice [180].

Human serum albumin (HSA): HSA is the major protein in plasma that carries several components such as metabolites, hormones and steroids in blood. It finds applications in drug delivery systems and vaccine production. Its recombinant expression in *P. pastoris* is advantageous because it overcomes the disadvantages of extraction based on collected human plasma. High-level expression in *P. pastoris* has resulted in 17.47 g/L of HSA. Cultivation at the fermenter level has resulted in productivity of 300 mg/L per day with reduced proteolysis. Since recombinant HSA is susceptible to impurities, a two-step purification strategy has been attempted to obtain a purified product. The first step involved partially unfolding the recombinant HSA to release the impurities, followed by crystallization [181–183].

Streptokinase (SK) is a thrombolytic agent that helps in the removal of blood clots. Obstruction in the blood flow leads to several disorders such as pulmonary embolism, deep-vein thrombosis and ischemic stroke. Streptokinase is considered as a vital agent that is considerably cost-effective and has a better half-life than other compounds like urokinase and tissue plasminogen activator. Since the heterologous

expression of streptokinase in *E. coli* leads to plasmid instability and cell toxicity, *P. pastoris* was explored as an alternate host. Adivitiya and co-workers expressed a 6× His-tagged and non-tagged rSK in *Pichia*, which led to 538 and 582 mg/L of protein production, respectively, at the shake-flask level. The bioreactor studies further improved it to 4.25 g/L, with a volumetric productivity of 57.43 mg/L/h [184]. The secretion efficiency of the PIR1 (protein with internal repeats) signal sequence was tested by targeting the secretion of rSK under the PIR1 signal sequence. The cell-retention strategy enhanced the product concentration to 3049.53 mg/L even at the shake flask level [37].

18.4.3 Enzyme Production in *P. pastoris*

18.4.3.1 Cellulase

Cellulases and hemicellulases are accountable for 20% of the industrial market at the global level. They hold applications in the food, feed, brewery and wine industries. Apart from these sectors, they are also in demand in the pulp and paper industry, in detergents, additives and also in pharmaceutical production. One of the major industrial segments requiring a high amount of cellulases is the lignocellulosic-based bioethanol industry. The enormous pressure on the depleting fossil fuels has resulted in this sector's growth that will help to generate cellulosic ethanol from plant-based resources. Cellulose is a complex polymer (crystalline/amorphous) comprising repeating residues of β-D-glucopyranose linked together by β-1,4 linkages. The dissolution of cellulose is an intricate process that requires the action of three cellulases: endoglucanase, which breaks down cellulose to release cello-oligosaccharides and glucose molecules, and cellobiohydrolase, which acts on the crystalline form of cellulose from its reducing and non-reducing ends to release cello-oligosaccharides and cellobiose. The third enzyme, β-glucosidase, further acts on cello-oligosaccharides and cellobiose to release glucose units [185].

Several reports show that cellulases of different source organisms have been successfully expressed using the *P. pastoris* system. Many are constitutively expressed under the *GAP* promoter. Endoglucanases of two different fungal origin, *Aspergillus fumigatus* (*Af*Cel12A) and *Thermoascus aurantiacus* (*Ta*Cel5A), were expressed in *P. pastoris*, using the PichiaPink™ expression system. The *Af*Cel12A production under the *GAP* promoter was similar or higher than the *AOX1* promoter [186]. In combination with *Trichoderma reesei* hydrophobin HFBII signal peptide, the *GAP* promoter successfully secreted mollusk *Ampullaria crossean* endoglucanase. Using the SMD1163 strain of *P. pastoris*, EG27I endoglucanase was secreted at a concentration of 47.7 mg/L at the bioreactor level with a specific activity of 26.8 U/mg [187]. D2-BGL is a β-glucosidase isolated from the fungus, *Chaetomella raphigera*, that showed enhanced substrate affinity when expressed in *P. pastoris*. Hence, attempts were made to further improve the catalytic efficiency of this enzyme via a random mutagenesis strategy. Initially, the wild-type enzyme was expressed in the recombinant hosts, *S. cerevisiae* and *P. pastoris*. The recombinant enzyme produced from *S. cerevisiae* was hyper-mannosylated and displayed lower

specific activity at 10 mM cellobiose (144.6 ± 20.2 U/mg) as compared to *P. pastoris*-expressed enzyme under the *GAP* promoter (214.3 ± 25.0 U/mg). Therefore, *S. cerevisiae* was used to prepare the mutant library for the enzyme D2-BGL, from which the mutant Mut M harbouring beneficial mutations were chosen. It was expressed in *P. pastoris* that exhibited higher V_{max}, enhanced expression (2.7-fold) and better substrate inhibition tolerance than the wild-type enzyme [188].

Thermostable cellulases are important in lignocellulosic biotechnology as they can be simultaneously employed with the saccharification process. They confer several advantages such as fast substrate hydrolysis and reduced risk of contamination. A thermostable cellulase was isolated from the lignocellulosic-degrading fungus, *Thermoascus aurantiacus*, RCKK and cloned in the pPICZαA vector. The overexpression studies revealed that the recombinant endoglucanase possessed high thermostability and retained 50% of its activity even when the enzyme was incubated at a temperature of 70 and 80 °C for 10 and 6 h, respectively [189]. β-Glucosidase can be used for transglycosylation reaction to synthesize sophorose from glucose. Sophorose is a potent inducer for the expression of cellulase genes that help overcome the drawbacks posed by the conventional inducer molecules such as Avicel and solka floc. Therefore, a β-glucosidase gene (*bgl*) was isolated from *Aspergillus niger* ZU07 and expressed in *P. pastoris* under the *AOX1* promoter. This resulted in the secretion of 129 IU/mL of the protein that successfully catalysed transglycosylation reaction, producing glucose–sophorose mixture (GSM) [190].

An innovative indirect yeast cell surface display technique was designed based on *P. pastoris*, where cells were engineered to display minicellulosomes on their surface. The strategy involved the participation of an ultra-high-affinity IM7/CL7 protein pair. The *S. cerevisiae* cell wall protein, SED1 was fused with the IM7 scaffoldins, and as a result, the fusion was displayed on the cell surface. Then, cellulases from different microbial origins (*Yarrowia lipolytica* cellobiohydrolase, *Clostridium thermocellum* DSM1237 endoglucanase, β-glucosidase *Thermoanaerobacterium thermosaccharolyticum* DSM 571 and *Thermobifda fusca* carbohydrate-binding module) were fused with CL7 protein and expressed in the heterologous host *E. coli*. The enzymes were purified and incubated with the engineered variants of *P. pastoris* displaying IM7 protein, leading to the in vitro assemblage of microcellulosomes over the cell surface. The engineered cells demonstrated the ability to utilize Avicel, phosphoric acid-swollen cellulose (PASC) and carboxymethyl cellulose (CMC), leading to the production of 2.5, 1.2 and 5.1 g/L of ethanol [191].

18.4.3.2 Xylanases

Xylanases are industrially relevant enzymes with applications in food and feed additives, paper-bleaching and biofuel production. Two major xylanases/hemicellulases required for xylan hydrolysis include endo-1,4-xylanases and β-xylosidases enzymes. The yeast system is suitable for xylanase production, as glycosylated enzymes exhibited better thermostability and resistance to harsh conditions. Kiribayeva and co-workers reported the *Bacillus sonorensis* T6 xylanase

production in *Pichia*. The glycosylated xylanase expressed in *Pichia* resulted in 40% higher thermostability than its counterpart from *E. coli*. The enzyme displayed activity in a wide range of pH with 100% activity at pH 3–11 after 10 h of incubation and 68% activity at pH 2.0 (after 1 h incubation). Under optimal conditions, *Pichia*-produced xylanase activity was recorded as 873.8 U/mg. Thus, recombinant xylanase's high thermal and pH stability makes it a useful enzyme in the feed industry [192]. Furthermore, Xia and co-workers designed a dual-plasmid system for the constitutive expression of *Streptomyces* sp. FA1 xylanase (XynA). They utilized two promoters: a GAP promoter and another having recently discovered constitutive GCW14 (cell wall protein) promoter. Among the different combinations tested, the combination containing the integrated GAP promoter and encompassing GCW14 episomally showed the highest production of xylanase. Hence, XynA expression using the constitutive dual plasmid methanol-free system reached the value of 13.6 g/L using the *Pichia* host [193].

Two novel xylanases, namely XYN10A_MALCI and XYN11A_MALCI, from thermostable *Malbranchea cinnamomea* were expressed in *P. pastoris*. The XYN11A_MALCI was expressed at a higher level of 573.32 U/mL with thermal stability at 70 °C and displayed active catalytic nature against the xylans. Further, the XYN11A_MALCI and XYN10A_MALCI were evaluated with commercial enzyme cellulase Cellic CTec2 to hydrolyse the pre-treated rice straw and bagasse. The synergistic effect of both the xylanases and the commercial cellulase was recognized as an effective strategy for hydrolysis of acid/alkali-treated lignocellulose biomass [194]. Recently, the gene coding for GH11 xylanase (from *Bacillus pumilus* SSP-34) was codon-optimized and expressed in the *Pichia* under the methanol-inducible AOX1 promoter. The GH11 xylanase characterization showed a high molecular weight due to glycosylation with improved stability over the native enzyme. The recombinant xylanase produced after submerged fermentation had 6000 U/mL activity after 120 h of cultivation. Thus, the recombinant GH11 xylanase holds the potential to be used in industrial-scale applications [195].

A double-plasmid co-expression method was developed to achieve high titers of endo-xylanase (XynC) GH10 from *Aspergillus niger*. The *P. pastoris* GS115 strain was transformed with one plasmid, resulting in its integration followed by the transformation and integration of another plasmid at a different locus, resulting in a 33% improvement in the yield of recombinant endo-xylanase. The bioprocess optimization resulted in 1650 U/mL of GH10 XynC, exhibiting maximum activity at 55 °C, pH 5.0. The recombinant enzyme showed stability in the pH range of 4.5–7.0, which was similar to the commercial cellulases, indicating the application of GH10 XynC from *A. niger* in industrial applications [196].

Karaoglan and co-workers compared the strengths of different promoters P_{AOX1}, P_{GAP} and P_{ADH3} to produce *Aspergillus niger* xylanase (XylB). Fermentation studies revealed the highest production of XylB under the control of ethanol-inducible P_{ADH3} at a level of 3725 U/mL compared to AOX1 (2095 U/mL) and GAP (580 U/mL). Thus, P_{ADH3} is a potential alternative for the high-level recombinant protein production in *Pichia* [25]. Further, the fermentation process was optimized to improve the production of industrially relevant thermoalkaliphilic xylanases.

Optimal glycerol feeding and four-step methanol feeding with a stepwise increase in feed rate improved product yields at the fermenter level. The xylanase was produced with a maximum activity of 591.2 U/mL and exhibited temperature stability in a range of 60–80 °C and pH of 8.0–11.0 [197].

18.4.3.3 Amylase

Amylase is a commercially important enzyme that degrades starch to release sugar molecules. Alpha-amylase (α-1,4-D-glucan glucanohydrolase and endoamylase) catalyses the cleavage of α-1,4-glycosidic bonds present within the long chains of starch to release sugars including glucose, maltose and dextrin. Starch hydrolytic enzymes have a substantial economic significance on several industries, including food, textile and pharmaceutics. Several microbes produce this enzyme, but major bottlenecks are low yields and high production costs. A thermostable α-amylase from the thermophilic bacteria *Geobacillus stearothermophilus* SR74 was expressed in *P. pastoris* GS115 to a level of 28.6 U/mL. The purified product showed a specific activity of 151.8 U/mg [198]. A maltohexaose-producing α-amylase AmyM native to *Corallococcus* sp. was expressed in *P. pastoris* GS115 under the regulation of *FLD1* promoter with a maximum production of 220 mg/L in a 50-L bioreactor [199].

Similarly, amylases from different fungi, such as *Rhizomucor miehei* and *Aspergillus oryzae*, were cloned and expressed in *P. pastoris*. Recombinant α-amylase (29,794.2 U/mL) produced in *Pichia* showed a broad specificity towards different substrates, including amylose, amylopectin, pullulan and cyclodextrins. On the other hand, the heterologous expression of *A. oryzae* S2 endogenous amylase AmyA resulted in the secretion levels of up to 72 U/mL. Due to N-glycosylation, the recombinant enzyme produced in *Pichia* displayed better thermostability properties than the native enzyme. The crude extract had a specific activity of 386.6 U/mg, which was high compared to other reported α-amylases from strains of *Bacillus* and *Aspergillus* [200, 201].

The methanol-inducible expression of the codon-optimized *Bacillus licheniformis* α-amylase gene in 5- and 50-L fermenters led to the enzyme activity of 8100 and 11,000 U/mL, respectively [202]. In some instances, it has been observed that an increase in the copy number of the gene can help in enhancing the protein expression levels. Therefore, a strategy based on producing multiple transformations and post-transformational vector amplification was undertaken successfully in *P. pastoris* to generate a recombinant clone containing eight copies of a chimeric amylase. The chimeric amylase consisted of the catalytic domain of *Bacillus acidicola* amylase and N- and C-terminus residues of *Geobacillus thermoleovorans* amylase. The recombinant product possessed the ability to act on raw starches releasing maltose and malto-oligosaccharides [203]. The antibiotic resistance genes are a potential hazard to the environment, affecting agriculture and animal husbandry. An α-amylase gene isolated from *B. licheniformis* possessing six glycosylation sites was cloned and expressed in *P. pastoris* without any antibiotic resistance gene. The recombinant yeast was cultivated in flasks that yielded 900 U/mL of the glycosylated product [204]. Huang and co-workers studied the effects of HAC1p protein, a UPR (unfolded protein response) activator, on a *Geobacillus* sp. 4j

α-amylase expressed in *P. pastoris*. A 12-copy integrant of the α-amylase gene resulted in the enzymatic activity of 305 U/mL. However, when the HAC1p protein was co-expressed along with α-amylase, the secretion levels of the protein increased with an increase in the gene copy number of HAC1p. The co-expression of six copies of HAC1p regulated by the *AOX1* promoter with α-amylase resulted in its enhanced activity of 2200 U/mL. Further, constitutive expression of the HAC1p protein with 17 copies improved the enzymatic activity to 3700 U/mL [205].

18.4.3.4 Lipases

Lipases are triacylglycerol hydrolases with the ability to hydrolyse fats and lipids. Lipases have a wide range of applications in industrial and biotechnological sectors such as in the feed additive industry, food processing, fine chemicals, detergents and biodiesel production. Therefore, a myriad of data is available on the high-level production of Lipases using *Pichia* as a host system. The thermo-active and alkaline Lipase from *Thermomyces dupontii* (TDL) was expressed in *Pichia*, under methanol-inducible and constitutive promoters. Among the different methanol-inducible promoters, the formaldehyde dehydrogenase promoter (P_{FLD1}) supported the highest expression of Lipase with 27,076 U/mL activity in a 5-L fermenter culture, while the glucose/glycerol-inducible constitutive glycosyl phosphatidyl inositol-anchored protein promoter (P_{GCW14}) resulted in maximum lipase expression at 17,535 U/mL. Thus, an alternative promoter system was documented that can direct the high production of industrially relevant TDL in *Pichia* [206].

A recent report carried out the cell surface expression of *Rhizomucor miehei* lipase (RML) on yeast cells using a PIR-1 anchor system. The optimal temperature of RML-PIR1 activity was 45 °C. The whole-cell biocatalyst was employed in the esterification reaction using soybean fatty acid distillate (SFAD, a by-product of soyabean oil) and palm fatty acid distillate (PFAD, a by-product of palm oil) as substrates for ester production to synthesize biodiesel. The highest conversion rate (79.1%) was observed with SFAD, with only 10% biocatalyst in the solvent-free medium. These conversion rate values were found to be 8 times higher than the commercially available RML. Further, the use of glutaraldehyde imparted high stability to the whole-cell biocatalyst, where the RML-PIR1 was able to retain 87.6% relative activity after the sixth cycle of reuse [207].

Tian and co-workers expressed the *Rhizomucor miehei* lipase (RML) in *Pichia* using crude glycerol as the carbon source. The impurities of crude glycerol (methanol, glycerol, grease, methyl ester, Na^+, Ca^{2+} and Fe^{3+}) increased the rate of *P. pastoris* entry into the stationary phase. The Na^+, Ca^{2+} metal ions and grease resulted in higher lipase activity. Optimization of these impurities concentration using response surface methodology resulted in 2.5 times improvement in lipase activity (1437 U/mL) compared to control. Thus, this strategy highlighted the importance of lipase production using crude glycerol without its pre-treatment with the help of *P. pastoris* GS115 [208].

Due to its lower expression levels, *Aspergillus*-produced Lipase has had limited industrial applications. To resolve the problem, the expression of *A. niger* Lipase (ANL) was carried out in *Pichia* GS115 in fusion with different fusion tags. The

SUMO-tagged acidic ANL showed higher activity of 960 ± 40 U/mL, with 1.85-fold enhancement than the non-tagged ANL. SUMO-ANL had the maximum activity at pH 2.5, was resistant to pepsin and was inhibited by bile salts, suggesting that it could also be a promising candidate for gastric digestion [209].

Abu and co-workers reported the production of *Geobacillus zalihae* thermostable lipase in novel *Pichia* sp. strain SO (*Pichia guilliermondii* strain SO of Malaysia origin). The medium conditions for the optimal production of T1 lipase were optimized using the response surface methodology. Plackett–Burman Design (PBD) showed that the inoculum size, temperature, agitation, culture volume and incubation time significantly impacted T1 lipase production. Box–Behnken Design (BBD) was employed to optimize the medium conditions. Hence, using PBD and BBD statistical models, different parameters were optimized (incubation time 39.33 h, temperature 31.76 °C, culture volume 132.19 mL, agitation speed 288.2 rpm and inoculum size 3.64%), resulting in a 3-fold increase in T1 lipase production (13.72 U/mL at $OD_{600} = 24.5$) [210].

In a recent study, the PGK1 promoter was employed for the constitutive expression of Lipase B from *Candida antarctica* (CalB) in *Pichia* using fed-batch and chemostat cultivation strategies. In continuous mode, the highest specific productivity and volumetric productivity were observed at the highest specific growth rate (0.16 h^{-1}), thereby resulting in 1.5- and 4.8-fold enhancement in volumetric and specific productivity, respectively, compared to fed-batch mode. Further, the continuous cultivation strategy was more suitable for long runs with 5.8 times greater production of CalB (1.89×10^8) after 6 weeks of cultivation over the fed-batch (3.24×10^7) [211].

Zhou and co-workers reported the production of the thermostable mutant of Lipase 2 from *Yarrowia lipolytica* (YlLip2) in *Pichia*. By gene dosage optimization, the activity of mutant YlLip2 was improved by 89%. Further, optimization of shake flask cultivation conditions resulted in YlLip2 production with enhanced activity (1465 U/mL). The co-expression of *Vitreoscilla stercoraria* haemoglobin (terminal oxidase) and Hac1p (yeast transcription factor) in YlLip2 mutant enhanced oxygen uptake efficiency and reduced ER stress. Fermentation studies in a 3-L fermenter led to 9080 U/mL YlLip2 production [212].

18.4.3.5 Phytase

Phytase enzyme is required to break down phytate into inorganic phosphate (Pi). Phytate is abundantly present in animal and human dietary products such as legumes, oilseeds and cereals. It is also referred to as an antinutrient as it readily forms stable complexes with proteins, carbohydrates and divalent metals ions, thereby leading to reduced bioavailability of essential nutrients. The phosphorous content in diets can be enhanced by providing Pi externally, which is an expensive approach. As an alternative strategy, phytase supplementation can help to improve nutrient availability. Hence, phytase holds immense potential in the feed and human food industry and animal waste treatment. Two thermostable phytases isolated from *Yersinia frederiksenii* and *Aspergillus niger* were codon-optimized and expressed in *P. pastoris*. The recombinant enzymes exhibited pH and temperature stabilities

[213]. Phytase is also a pH and temperature-sensitive enzyme. Therefore, a codon-optimized phytase gene of a thermophilic mold *Sporotrichum thermophile* was expressed in *P. pastoris* to a level of 480 ± 23 U/mL, approximately 40-fold higher than shake-flask results [214]. Recombinant *Pichia* strains were developed that harboured expression cassette, consisting of *S. thermophile* phytase and *Bacillus acidicola* alpha-amylase genes regulated under the control of both *GAP* and *AOX* promoters. In the phytase construct, the strain consisted of five copies of the phytase gene, two and three under *GAP* and *AOX* promoters, respectively. The amylase and phytase-producing strains under the dual promoters showed an enhanced activity by 1.8- and 1.3-fold over the *AOX* promoter [215]. Yang and co-workers observed that the recombinant expression of lily pollen's alkaline phytase secreted from *P. pastoris* under the *GAP* promoter displayed an 8-fold enhanced activity (75–80 mg/L) as compared to *AOX* promoter (8–10 mg/L) [216]. Different strategies have been explored to improve recombinant phytase expression. Modification of *AOX1* promoter, α-secretion signal, enhancing gene copy number, expressing Hac1p protein required for proper folding and secretion of the protein, overexpression of PDI (disulphide isomerase) gene and the use of Pep4 (proteinase A) knock-out strains are some of the approaches that have been used to improve phytase production [217, 218].

18.4.3.6 Laccase

Laccases are copper-containing oxidases that catalyse the oxidation of several organic and inorganic substances that have abundant electrons. Some of these compounds include aromatic and aliphatic amines, diamines and polyphenols. Molecular oxygen is the electron acceptor that releases water as the sole by-product. Redox mediators further cause the expansion of the substrate range to non-phenolic lignin products. One of laccases' major industrial and environmental applications is dye decolourization in industrial and textile wastewater treatment. They have emerging applications in bioremediation, biomass breakdown and the development of biofuel cells. A novel laccase from the basidiomycete, *Moniliophthora roreri*, was expressed in the fed-batch culture of *P. pastoris*, resulting in high yields of the protein of approximately 1.05 g/L. The recombinant enzyme Mrl2 displayed a high enzyme value of 280,000 U/L. Another laccase of fungal origin, Lcc9 (*Coprinopsis cinerea*), was expressed in *P. pastoris* GS115, where a high enzyme activity of 3138 ± 62 U/L was recorded. Further, the efficacy of the enzyme in dye decolourization of indigo carmine and azo dyes were tested [219, 220].

Since the industrial wastewater comprises several compounds such as chlorides, detergents, sulphates and metal ions and maintains a neutral or alkaline pH, it is expected that the laccases should remain stable under these conditions. A gene of *Thermus thermophilus* SG0.5JP17-16 (*LacTT*) possessing putative laccase properties was successfully cloned and expressed in *P. pastoris*, producing 6130 U/L of the enzyme in a 10-L fermenter. The enzyme displayed stability towards a wide range of pH, temperature and salts that retained the ability to decolourize dyes such as Congo Red and Reactive Black B [221]. Varied

combinatorial techniques based on side-directed mutagenesis, codon-optimization and changing cultivation conditions, helped in improving *Bacillus licheniformis* LSO4 laccase in *P. pastoris*. A D500G mutant of laccase, expressed in *P. pastoris*, displayed 2.1 times higher activity. The mutant enzyme exhibited a superior activity of 208 ± 8 U/L without the external addition of copper compared to the wild-type enzyme (<5 U/L) when expressed in *P. pastoris* [222].

18.4.3.7 Other Enzymes

Several enzymes have been successfully expressed using *P. pastoris*. Some of these proteins include proteases, pectinase and α-galactosidase, which possess applications in different industries. Since the discussion of all these recombinant enzymes is beyond this chapter's scope, a few examples of recombinant expression of these enzymes in *P. pastoris* are described below.

A proteinase K native to *Tritirachium album Limber* was cloned in *Pichia* via multi-expression vectors, as the expression levels increase with an increase in the gene copy number. The enzyme is a serine endoproteinase that forms biomolecules by catalysing the degradation of cellular constituents, helps in the removal of DNAases and RNAases and serves as a detergent supplement. Its ability to degrade keratin makes it a suitable candidate to treat keratin-containing products from the poultry industry. A high expression of 8.069 mg/mL and enzymatic activity of 108,295 U/mL were obtained from a five-copy strain of *P. pastoris* after 84 h of fermentation [223]. Subtilisin, a serine alkaline protease isolated from *Melghiribacillus thermohalophilus* Nari2A^T, was cloned in *E. coli* and *P. pastoris* expression vectors, where *Pichia* showed its efficient expression [224]. Similarly, collagenolytic aspartic protease isolated from *Thermomucor indicae seudaticae* was expressed in both bacterial and yeast hosts *E. coli* and *P. pastoris*. Although the maximum activity of both expressed enzymes was at pH 5, the *Pichia*-expressed enzyme exhibited a higher thermostability [225]. Further, high enzymatic activities of 3480.4 and 49,370 U/mL of *Rhizomucor miehei* aspartic protease and thermolysin-like protease from *Aspergillus oryzae* were obtained using *P. pastoris* expression system [226, 227].

Pectinases are another group of enzymes that have applications in the food and agriculture industries. It is required for different processes, such as clarifying fruit juice, saccharifying lignocellulosic biomass and extracting vegetable oil. Pectin hydrolases, comprising polygalacturonase (PG) and polymethylgalacturonase (PMG), catalyse the hydrolysis of α-1,4 glycosidic linkages in pectic acid and pectin. Due to its commercial importance, it has been successfully expressed in *P. pastoris*. A comparative expression of the endo-polygalacturonase (PGL) gene (codon-optimized and native) endogenous to *A. niger* was carried out in *P. pastoris* under the regulation of two different promoters, *AOX1* and the ethanol-inducible *ADH2*. The codon-optimized gene under the *ADH2* promoter yielded the best enzymatic activity of 42.33 U/mL [228]. Similarly, secretory expression of *A. aculeatus* endo-polygalacturonase (Endo-PG) was targeted in *P. pastoris* KM71 strain, resulting in a specific activity of 1892.08 U/mg on citrus pectin. This acidic Endo-PG had the

potential to participate in an eco-friendly bio-scouring as compared to the toxic chemical scouring process [229].

18.4.4 Commercial Products Based on *P. pastoris*

18.4.4.1 Jetrea (Ocriplasmin)

Ocriplasmin, also known by its trade name Jetrea (ThromboGenics NV, Leuven, Belgium), is a recombinant serine protease expressed using the *Pichia pastoris* expression system. It is used to treat symptomatic vitreomacular adhesion that causes the formation of macular holes, edema and macular distortion. As a solution, pharmacological vitreolysis is used to treat this condition. Since fibronectin and laminin are majorly responsible for maintaining the vitreomacular adhesion, plasmin is utilized in the preoperative stage to initiate posterior vitreous detachment while performing a vitrectomy. The autolytic instability of plasmin makes it an unsuitable treatment strategy. Therefore, it led to the development of the recombinant enzyme, microplasmin or ocriplasmin, having the same catalytic features with better stability. Ocriplasmin is a truncated version of the serine protease plasmin, which is a potent collagenase activator with proteolytic activities against the structural components of the vitreoretinal interface, laminin and fibronectin. It is a small protein compared with plasmin, which helps it penetrate the tissues with excellent efficacy. It comprises two polypeptides of 230 and 19 amino acid residues linked together by two disulphide bonds. The enzyme works by a two-stage process, catalysing vitreous liquefaction and vitreoretinal separation. Ocriplasmin has gained marketing approval in Europe and the United States, which stands as the sole licensed non-surgical medical intervention for symptomatic vitreomacular adhesion. The administration dose comprises 125 μg product in 100 μL as a one-time intravitreal injection. Further, the autolytic and proteolytic properties of ocriplasmin were improved using site-directed mutagenesis. The mutants were developed and expressed using the *P. pastoris* expression system [230–232].

18.4.4.2 Kalbitor (Ecallantide)

Hereditary angioedema is an autosomal dominant swelling disorder that causes the skin and mucosal tissues to swell. This leads to a decline in the vascular integrity and movement of fluids into the interstitial regions. The condition arises due to the lack of a functional C1-inhibitor that acts against the plasmin kallikrein–kinin, coagulation and complement mechanisms. The deficiency leads to swelling in the face, genital and gastrointestinal tracts. Further, the inflammation of the laryngeal region is predominantly a matter of concern as it can lead to asphyxiation. One of the treatment measures that the FDA approved in 2009 is the administration of ecallantide (kalbitor). Kalbitor (Dyax Corp, Burlington, MA) is a 60-residue-long recombinant protein inhibitor of human plasma kallikrein expressed in *P. pastoris*. Ecallantide works by binding and blocking the plasma kallikrein binding site, thereby causing reduced production of bradykinin. It has been studied that bradykinin, a vasodilator, is the central mediator of the attacks caused due to hereditary

angioedema. The drug could be administered to patients over 12 years of age where subcutaneous dosing of 30 mg is within the recommended limits [233–235].

18.4.4.3 Semglee (MYL1501D Insulin Glargine) and Insugen (Biocon)

Semglee® (Mylan), a long-acting human insulin analogue, is a biosimilar to insulin glargine 100 U/mL (Lantus®, Sanofi), which improves peripheral glucose utilization and lowers blood glucose levels. The FDA approved this biosimilar as a therapy for type 1 diabetes in children and adults and type 2 in adults. It is manufactured via recombinant DNA technology in *P. pastoris*, where its physicochemical properties are similar to that of the EU- and US-sourced insulin glargine 100 U/mL. The immunogenic studies have revealed that it has features comparable to the reference insulin in patients suffering from type 1 and type 2 diabetes. Further, in trials which tested its efficacy, it showed an equivalent glycaemic efficacy even while substituting the reference insulin glargine with the biosimilar Semglee®. Its approval for use has been given in the EU for patients of or more than 2 years of age suffering from diabetes mellitus and Japan where insulin therapy is recommended. Further, this is an economical alternative to Lantus®, whose 10 mL vials can cost up to US$ 283.60, as the biosimilar's 10 mL vial is priced at US$ 98.70 [236, 237].

Insugen® (Biocon Ltd., Bengaluru, India) is an insulin biosimilar synthesized in a *P. pastoris* expression system and is accessible as a reusable pen. It has been marketed in India since 2004 and Nigeria since 2010. In 2012, a phase 3 study in Europe indicated that it was at par with the Novo Nordisk's product's efficiency and safety [238, 239].

18.4.4.4 Shanvac-B (Hepatitis B Vaccine)

Hepatitis B is a severe communicable liver infection that affects 40 million people worldwide. Among the Indian population, 4% are carriers, and about 100,000 Indians die annually due to this disease. The two vaccine giants, Merck and GlaxoSmithKline, led the way in developing recombinant vaccines in 1986, and the prices rose to US$ 23 per dose. In India, plasma-derived vaccines were produced, but the risk of the spread of blood-borne diseases and the dependence on the donor highlighted the need to develop recombinant vaccines. Shanvac-B was the first recombinant vaccine against Hepatitis B developed in India. Developed in the yeast *P. pastoris* in 1997, it was introduced at US$ 1 per dose. The efficacy studies of this vaccine revealed that this Indian vaccine was safe to use and provided sufficient titers against Hepatitis B. Further comparative studies with other Hepatitis B vaccines such as GeneVac-B (Serum Institute of India Ltd., Pune) and Engerix B® (SmithKline Beecham Biologicals, Belgium), it has been concluded that there is no substantial variation between all the three vaccines [240–242].

18.4.4.5 Shanferon (Interferon)

Interferons are an essential class of signalling molecules characterized based on the type of receptor interactions. Interferon-α 2b (IFN-α2b) is the type I interferon molecule that is used as a medical intervention for hepatitis B and C infections.

The recombinant IFN-α2b is expressed in the form of a single polypeptide chain comprising 165 amino acid residues. With a molecular weight of 19.2 kDa, it was first marketed as Intron A by Schering Corp (Merck) in 1986, expressed using *E. coli*. The Shantha Biotechnics India is credited with manufacturing recombinant IFN-α2b (Shanferon) on the basis of *Pichia pastoris*-based expression system. It was the first report of the commercial synthesis of this therapeutic protein in the yeast system. Shanferon was reportedly economically priced at US$ 6.50, which is lower than the imported cost of US$ 26. Further, this *Pichia*-produced molecule has shown biological and chemical similarities with the National Institute for Biological Standards and Control (NIBSC, UK) international standard [241, 243, 244].

18.5 Summary and Conclusions

Pichia pastoris has emerged as a promising platform for producing commercially and therapeutically relevant biomolecules. Being a eukaryotic host, it is one of the most suitable expression hosts for recombinant proteins, requiring post-translational modifications and proper polypeptide folding for activity and stability. Further, it possesses several advantages over the most widely used yeast, *S. cerevisiae*, as it lacks the immunogenic terminal α-1,3 linkages on its core oligosaccharides. Additionally, its ability to glycosylate biomolecules in a more humanized manner makes it the system of choice for heterologous expression of therapeutic molecules. Further, its preference for the aerobic mode of respiration helps achieving high cell density at the bioreactor level. As a result, researchers have focused on this methylotrophic yeast to develop novel vectors, signal sequences and promoters that will enable the synthesis of several value-added products. Various biopharmaceuticals and proteins of industrial relevance have been expressed in *Pichia* on a commercial scale, further highlighting its potential as a robust and dependable expression system. The ongoing research related to its utility as a model system for protein expression and scale-up strategies at the fermenter level is a promising step towards developing an effective eukaryotic expression host for a range of products.

References

1. Kaur J, Kumar A, Kaur J (2018) Strategies for optimization of heterologous protein expression in *E. coli*: roadblocks and reinforcements. Int J Biol Macromol 106:803–822
2. Baeshen MN et al (2015) Production of biopharmaceuticals in *E. coli*: current scenario and future perspectives. J Microbiol Biotechnol 25:953–962
3. Duman-Ozdamar ZE, Binay B (2021) Production of industrial enzymes via *Pichia pastoris* as a cell factory in bioreactor: current status and future aspects. Protein J 40:367–376
4. de Sa Magalhaes S, Keshavarz-Moore E (2021) *Pichia pastoris* (*Komagataella phaffii*) as a cost-effective tool for vaccine production for low- and middle-income countries (LMICs). Bioengineering (Basel) 8:119

5. Love KR, Dalvie NC, Love JC (2018) The yeast stands alone: the future of protein biologic production. Curr Opin Biotechnol 53:50–58
6. Walsh G (2018) Biopharmaceutical benchmarks 2018. Nat Biotechnol 36:1136–1145
7. Gasser B, Mattanovich D (2018) A yeast for all seasons - is *Pichia pastoris* a suitable chassis organism for future bioproduction? FEMS Microbiol Lett 365. https://doi.org/10.1093/femsle/fny181
8. Baghban R et al (2019) Yeast expression systems: overview and recent advances. Mol Biotechnol 61:365–384
9. Malik A et al (2021) A review on *Pichia pastoris*: a successful tool for expression of recombinant proteins. Pharma Innov SP-10(11):550–556
10. Rebello S et al (2018) Non-conventional yeast cell factories for sustainable bioprocesses. FEMS Microbiol Lett 365. https://doi.org/10.1093/femsle/fny222
11. Darby RA et al (2012) Which yeast species shall I choose? *Saccharomyces cerevisiae* versus *Pichia pastoris* (review). Methods Mol Biol 866:11–23
12. Karbalaei M, Rezaee SA, Farsiani H (2020) *Pichia pastoris*: a highly successful expression system for optimal synthesis of heterologous proteins. J Cell Physiol 235:5867–5881
13. Vittaladevaram V (2021) A short communication on *Pichia pastoris* vs. *E. coli*: efficient expression system. Ann Proteom Bioinform 5:049–050
14. Cos O et al (2006) Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: a review. Microb Cell Factories 5:17
15. De Schutter K et al (2009) Genome sequence of the recombinant protein production host *Pichia pastoris*. Nat Biotechnol 27:561–566
16. Cereghino JL, Cregg JM (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol Rev 24:45–66
17. Mastropietro G, Aw R, Polizzi KM (2021) Expression of proteins in *Pichia pastoris*. Methods Enzymol 660:53–80
18. Puxbaum V, Mattanovich D, Gasser B (2015) Quo vadis? The challenges of recombinant protein folding and secretion in *Pichia pastoris*. Appl Microbiol Biotechnol 99:2925–2938
19. Li P et al (2007) Expression of recombinant proteins in *Pichia pastoris*. Appl Biochem Biotechnol 142:105–124
20. Juturu V, Wu JC (2018) Heterologous protein expression in *Pichia pastoris*: latest research progress and applications. Chembiochem 19:7–21
21. Zhang AL et al (2009) Recent advances on the GAP promoter derived expression system of *Pichia pastoris*. Mol Biol Rep 36:1611–1619
22. Vogl T, Glieder A (2013) Regulation of *Pichia pastoris* promoters and its consequences for protein production. New Biotechnol 30:385–404
23. Duan H et al (2009) Both the *AOX1* promoter and the *FLD1* promoter work together in a *Pichia pastoris* expression vector. World J Microbiol Biotechnol 25:1779–1783
24. Koller A, Valesco J, Subramani S (2000) The CUP1 promoter of *Saccharomyces cerevisiae* is inducible by copper in *Pichia pastoris*. Yeast 16:651–656
25. Karaoglan M, Karaoglan FE, Inan M (2016) Comparison of *ADH3* promoter with commonly used promoters for recombinant protein production in *Pichia pastoris*. Protein Expr Purif 121: 112–117
26. Karaoglan M, Karaoglan FE, Inan M (2016) Functional analysis of alcohol dehydrogenase (*ADH*) genes in *Pichia pastoris*. Biotechnol Lett 38:463–469
27. Lin-Cereghino J et al (2008) Direct selection of *Pichia pastoris* expression strains using new G418 resistance vectors. Yeast 25:293–299
28. Sunga AJ, Cregg JM (2004) The *Pichia pastoris* formaldehyde dehydrogenase gene (*FLD1*) as a marker for selection of multicopy expression strains of *P. pastoris*. Gene 330:39–47
29. Wan H et al (2004) A new dominant selection marker for transformation of *Pichia pastoris* to soraphen A resistance. J Microbiol Methods 57:33–39

30. Yang J et al (2014) Hygromycin-resistance vectors for gene expression in *Pichia pastoris*. Yeast 31:115–125
31. Lin Cereghino GP et al (2001) New selectable marker/auxotrophic host strain combinations for molecular genetic manipulation of *Pichia pastoris*. Gene 263:159–169
32. Nett JH et al (2005) Cloning and disruption of the *Pichia pastoris ARG1*, *ARG2*, *ARG3*, *HIS1*, *HIS2*, *HIS5*, *HIS6* genes and their use as auxotrophic markers. Yeast 22:295–304
33. Thor D et al (2005) Cloning and characterization of the *Pichia pastoris MET2* gene as a selectable marker. FEMS Yeast Res 5:935–942
34. Ahmad M et al (2014) Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. Appl Microbiol Biotechnol 98:5301–5317
35. Lin-Cereghino GP et al (2013) The effect of alpha-mating factor secretion signal mutations on recombinant protein expression in *Pichia pastoris*. Gene 519:311–317
36. Neiers F et al (2021) Comparison of different signal peptides for the efficient secretion of the sweet-tasting plant protein Brazzein in *Pichia pastoris*. Life (Basel) 11. https://doi.org/10.3390/life11010046
37. Adivitiya et al (2019) Development of a streptokinase expression platform using the native signal sequence of the protein with internal repeats 1 (PIR1) in *P. pastoris*: gene dosage optimization and cell retention strategies. Process Biochem 83:64–76
38. Khasa YP et al (2011) Isolation of *Pichia pastoris PIR* genes and their utilization for cell surface display and recombinant protein secretion. Yeast 28:213–226
39. Aw R et al (2017) Expressing anti-HIV VRC01 antibody using the murine IgG1 secretion signal in *Pichia pastoris*. AMB Express 7:70
40. Damasceno LM, Huang CJ, Batt CA (2012) Protein secretion in *Pichia pastoris* and advances in protein production. Appl Microbiol Biotechnol 93:31–39
41. Babavalian H et al (2016) Cloning and expression of recombinant human platelet-derived growth factor-BB in *Pichia Pink*. Cell Mol Biol (Noisy-le-Grand) 62:45–51
42. Elnaz A et al (2016) Expression of a recombinant therapeutic protein, lactoferrin, in PichiaPink™: a powerful antimicrobial protein. Biol Forum 8:471–478
43. Li B et al (2011) A novel protein expression system-PichiaPink™-and a protocol for fast and efficient recombinant protein expression. Afr J Biotechnol 10:19464–19472
44. Li Z et al (2021) Efficient improvement of surface displayed lipase from *Rhizomucor miehei* in PichiaPink™ protease-deficient system. Protein Expr Purif 180:105804
45. Katla S et al (2019) Novel glycosylated human interferon alpha 2b expressed in glycoengineered *Pichia pastoris* and its biological activity: N-linked glycoengineering approach. Enzym Microb Technol 128:49–58
46. Effer B et al (2019) L-Asparaginase from *E. chrysanthemi* expressed in Glycoswitch®: effect of his-tag fusion on the extracellular expression. Prep Biochem Biotechnol 49:679–685
47. Liu CP et al (2018) Glycoengineering of antibody (Herceptin) through yeast expression and in vitro enzymatic glycosylation. Proc Natl Acad Sci U S A 115:720–725
48. Krainer FW et al (2012) Recombinant protein expression in *Pichia pastoris* strains with an engineered methanol utilization pathway. Microb Cell Factories 11:22
49. Kumar S, Mannil A, Mutturi S (2020) Modified chemical method for efficient transformation and diagnosis in *Pichia pastoris*. Protein Expr Purif 174:105685
50. Yamunasri P, Priyadharshini R, Uthandi S (2021) Evaluation of efficient transformation method for xylose reductase gene integration in *Pichia pastoris* GS115. Madras Agric J 107:1
51. Dagar VK, Khasa YP (2018) Combined effect of gene dosage and process optimization strategies on high-level production of recombinant human interleukin-3 (hIL-3) in *Pichia pastoris* fed-batch culture. Int J Biol Macromol 108:999–1009
52. Sunga AJ, Tolstorukov I, Cregg JM (2008) Posttransformational vector amplification in the yeast *Pichia pastoris*. FEMS Yeast Res 8:870–876
53. Portela RM et al (2017) Synthetic core promoters as universal parts for fine-tuning expression in different yeast species. ACS Synth Biol 6:471–484

54. Berg L et al (2013) Combinatorial mutagenesis and selection to understand and improve yeast promoters. Biomed Res Int 2013:926985
55. Hartner FS et al (2008) Promoter library designed for fine-tuned gene expression in *Pichia pastoris*. Nucleic Acids Res 36:e76
56. Ata Ö et al (2017) Transcriptional engineering of the glyceraldehyde-3-phosphate dehydrogenase promoter for improved heterologous protein production in *Pichia pastoris*. Biotechnol Bioeng 114:2319–2327
57. Xuan Y et al (2009) An upstream activation sequence controls the expression of AOX1 gene in *Pichia pastoris*. FEMS Yeast Res 9:1271–1282
58. Staley CA et al (2012) Analysis of the 5′ untranslated region (5′ UTR) of the alcohol oxidase 1 (AOX1) gene in recombinant protein expression in *Pichia pastoris*. Gene 496:118–127
59. Yang J et al (2018) Controlling AOX1 promoter strength in *Pichia pastoris* by manipulating poly (dA: dT) tracts. Sci Rep 8:1–11
60. Wang X et al (2016) PpNrg1 is a transcriptional repressor for glucose and glycerol repression of AOX1 promoter in methylotrophic yeast *Pichia pastoris*. Biotechnol Lett 38:291–298
61. Wang X et al (2016) Mit1 transcription factor mediates methanol signaling and regulates the alcohol oxidase 1 (AOX1) promoter in *Pichia pastoris*. J Biol Chem 291:6245–6261
62. Fischer JE, Glieder A (2019) Current advances in engineering tools for *Pichia pastoris*. Curr Opin Biotechnol 59:175–181
63. Vogl T et al (2014) Synthetic core promoters for *Pichia pastoris*. ACS Synth Biol 3:188–191
64. Näätsaari L et al (2012) Deletion of the *Pichia pastoris* KU70 homologue facilitates platform strain generation for gene expression and synthetic biology. PLoS One 7:e39720
65. Ito Y et al (2020) Exchange of endogenous and heterogeneous yeast terminators in *Pichia pastoris* to tune mRNA stability and gene expression. Nucleic Acids Res 48:13000–13012
66. Weninger A et al (2016) Combinatorial optimization of CRISPR/Cas9 expression enables precision genome engineering in the methylotrophic yeast *Pichia pastoris*. J Biotechnol 235: 139–149
67. Madhavan A et al (2021) Customized yeast cell factories for biopharmaceuticals: from cell engineering to process scale up. Microb Cell Factories 20:1–17
68. Yang Y et al (2020) High efficiency CRISPR/Cas9 genome editing system with an eliminable episomal sgRNA plasmid in *Pichia pastoris*. Enzym Microb Technol 138:109556
69. Cai P et al (2021) Recombination machinery engineering facilitates metabolic engineering of the industrial yeast *Pichia pastoris*. Nucleic Acids Res 49:7791–7805
70. Hirz M et al (2013) A novel cholesterol-producing *Pichia pastoris* strain is an ideal host for functional expression of human Na, K-ATPase α3β1 isoform. Appl Microbiol Biotechnol 97: 9465–9478
71. Bretthauer RK (2003) Genetic engineering of *Pichia pastoris* to humanize N-glycosylation of proteins. Trends Biotechnol 21:459–462
72. Choi B-K et al (2003) Use of combinatorial genetic libraries to humanize N-linked glycosylation in the yeast *Pichia pastoris*. Proc Natl Acad Sci U S A 100:5022–5027
73. Hamilton SR et al (2003) Production of complex human glycoproteins in yeast. Science 301: 1244–1246
74. Jacobs PP et al (2009) Engineering complex-type N-glycosylation in *Pichia pastoris* using GlycoSwitch technology. Nat Protoc 4:58–70
75. Hamilton SR et al (2013) Production of sialylated O-linked glycans in *Pichia pastoris*. Glycobiology 23:1192–1203
76. Peña DA et al (2018) Metabolic engineering of *Pichia pastoris*. Metab Eng 50:2–15
77. Duan G et al (2019) Screening endogenous signal peptides and protein folding factors to promote the secretory expression of heterologous proteins in *Pichia pastoris*. J Biotechnol 306:193–202
78. Weinhandl K et al (2016) *Pichia pastoris* mutants as host strains for efficient secretion of recombinant branched chain aminotransferase (BCAT). J Biotechnol 235:84–91

79. Heiss S et al (2013) Identification and deletion of the major secreted protein of *Pichia pastoris*. Appl Microbiol Biotechnol 97:1241–1249
80. Heiss S et al (2015) Multistep processing of the secretion leader of the extracellular protein Epx1 in *Pichia pastoris* and implications for protein localization. Microbiology 161:1356–1368
81. Gao J, Jiang L, Lian J (2021) Development of synthetic biology tools to engineer *Pichia pastoris* as a chassis for the production of natural products. Synth Syst Biotechnol 6:110–119
82. Vogl T et al (2016) A toolbox of diverse promoters related to methanol utilization: functionally verified parts for heterologous pathway expression in *Pichia* pastoris. ACS Synth Biol 5:172–186
83. Prielhofer R et al (2017) GoldenPiCS: a Golden Gate-derived modular cloning system for applied synthetic biology in the yeast *Pichia pastoris*. BMC Syst Biol 11:1–14
84. Nocon J et al (2014) Model based engineering of *Pichia pastoris* central metabolism enhances recombinant protein production. Metab Eng 24:129–138
85. Dragosits M, Mattanovich D (2013) Adaptive laboratory evolution—principles and applications for biotechnology. Microb Cell Factories 12:64
86. Moser JW et al (2017) Implications of evolutionary engineering for growth and recombinant protein production in methanol-based growth media in the yeast *Pichia pastoris*. Microb Cell Factories 16:1–16
87. Sarkari P et al (2017) An efficient tool for metabolic pathway construction and gene integration for *Aspergillus niger*. Bioresour Technol 245:1327–1333
88. Wang J et al (2017) Methanol-independent protein expression by AOX1 promoter with trans-acting elements engineering and glucose-glycerol-shift induction in *Pichia pastoris*. Sci Rep 7:1–12
89. Shen W et al (2016) A novel methanol-free *Pichia pastoris* system for recombinant protein expression. Microb Cell Factories 15:1–11
90. Mombeni M et al (2020) pMOX: a new powerful promoter for recombinant protein production in yeast *Pichia pastoris*. Enzym Microb Technol 139:109582
91. Kashyap A et al (2022) Development of a novel *Pichia pastoris* expression platform via genomic integration of lipase gene for sustained release of methanol from methyloleate. Prep Biochem Biotechnol:1–12. https://doi.org/10.1080/10826068.2022.2039941
92. Han L et al (2018) Redesigning of microbial cell surface and its application to whole-cell biocatalysis and biosensors. Appl Biochem Biotechnol 185:396–418
93. Kuroda K, Ueda M (2013) Arming Technology in Yeast-Novel Strategy for whole-cell biocatalyst and protein engineering. Biomol Ther 3:632–650
94. Andreu C, Del Olmo ML (2018) Yeast arming systems: pros and cons of different protein anchors and other elements required for display. Appl Microbiol Biotechnol 102:2543–2561
95. Zhang L et al (2013) Screening for glycosylphosphatidylinositol-modified cell wall proteins in *Pichia pastoris* and their recombinant expression on the cell surface. Appl Environ Microbiol 79:5519–5526
96. Li W et al (2015) Cell surface display and characterization of *Rhizopus oryzae* lipase in *Pichia pastoris* using Sed1p as an anchor protein. Curr Microbiol 71:150–155
97. Su GD, Zhang X, Lin Y (2010) Surface display of active lipase in *Pichia pastoris* using Sed1 as an anchor protein. Biotechnol Lett 32:1131–1136
98. Wang Q et al (2007) Construction of a novel system for cell surface display of heterologous proteins on *Pichia pastoris*. Biotechnol Lett 29:1561–1566
99. Spohner SC et al (2015) Expression of enzymes for the usage in food and feed industry with *Pichia pastoris*. J Biotechnol 202:118–134
100. Weinacker D et al (2013) Applications of recombinant *Pichia pastoris* in the healthcare industry. Braz J Microbiol 44:1043–1048
101. Khan MA et al (2017) Bioactivity studies of *Huh-7* cells derived human epidermal growth factor expressed in *Pichia pastoris*. Biosci Biotechnol Biochem 81:1114–1119

102. Khan MA et al (2014) Expression line approach to recombinant human epidermal growth factor into the yeast, *Pichia pastoris* from *Huh-7* cell line. Mol Biol Rep 41:1445–1451
103. Ornitz DM, Itoh N (2015) The fibroblast growth factor signaling pathway. Wiley Interdiscip Rev Dev Biol 4:215–266
104. Mu X et al (2008) High-level expression, purification, and characterization of recombinant human basic fibroblast growth factor in *Pichia pastoris*. Protein Expr Purif 59:282–288
105. Song Y et al (2016) Expression and purification of FGF21 in *Pichia pastoris* and its effect on fibroblast-cell migration. Mol Med Rep 13:3619–3626
106. Sadeghi S et al (2021) Keratinocyte growth factor in focus: a comprehensive review from structural and functional aspects to therapeutic applications of palifermin. Int J Biol Macromol 191:1175–1190
107. Bahadori Z, Kalhor HR, Mowla SJ (2018) Producing functional recombinant human keratinocyte growth factor in *Pichia pastoris* and investigating its protective role against irradiation. Enzym Microb Technol 111:12–20
108. Maity N et al (2016) Expression and control of codon-optimized granulocyte colony-stimulating factor in *Pichia pastoris*. Appl Biochem Biotechnol 178:159–172
109. Sigar M, Maity N, Mishra S (2017) Enhancing granulocyte colony-stimulating factor expression in *Pichia pastoris* through fusion with human serum albumin. Prep Biochem Biotechnol 47:364–370
110. Aggarwal S, Mishra S (2020) Differential role of segments of alpha-mating factor secretion signal in *Pichia pastoris* towards granulocyte colony-stimulating factor emerging from a wild type or codon optimized copy of the gene. Microb Cell Factories 19:199
111. Khasa YP et al (2007) Kinetic studies of constitutive human granulocyte-macrophage colony stimulating factor (hGM-CSF) expression in continuous culture of *Pichia pastoris*. Biotechnol Lett 29:1903–1908
112. Pal Y, Khushoo A, Mukherjee KJ (2006) Process optimization of constitutive human granulocyte-macrophage colony-stimulating factor (hGM-CSF) expression in *Pichia pastoris* fed-batch culture. Appl Microbiol Biotechnol 69:650–657
113. Wu J et al (2003) Combined use of *GAP* and *AOX1* promoter to enhance the expression of human granulocyte-macrophage colony-stimulating factor in *Pichia pastoris*. Enzym Microb Technol 33:453–459
114. Li H et al (2013) High level expression, efficient purification and bioactivity assay of recombinant human platelet-derived growth factor AA dimer (PDGF-AA) from methylotrophic yeast *Pichia pastoris*. Protein Expr Purif 91:221–227
115. Zhu RY et al (2012) Expression and purification of recombinant human serum albumin fusion protein with VEGF165b in *Pichia pastoris*. Protein Expr Purif 85:32–37
116. Arjmand S et al (2019) Enhancing chimeric hydrophobin II-vascular endothelial growth factor A_{165} expression in *Pichia pastoris* and its efficient purification using hydrophobin counterpart. Int J Biol Macromol 139:1028–1034
117. Liu ZM et al (2005) Secretory expression and characterization of a recombinant-deleted variant of human hepatocyte growth factor in *Pichia pastoris*. World J Gastroenterol 11: 7097–7103
118. Li P et al (2016) High-level secretory expression and purification of recombinant human interleukin 1 beta in *Pichia pastoris*. Protein Pept Lett 23:763–769
119. Wang S et al (2013) Expression, identification and characterize of CD25-binding epitope modified human IL-2 in *Pichia pastoris*. Indian J Microbiol 53:283–287
120. Lei J et al (2012) Expression, purification and characterization of recombinant human interleukin-2-serum albumin (rhIL-2-HSA) fusion protein in *Pichia pastoris*. Protein Expr Purif 84: 154–160
121. Dagar VK, Adivitiya, Devi N, Khasa YP (2016) Bioprocess development for extracellular production of recombinant human interleukin-3 (hIL-3) in *Pichia pastoris*. J Ind Microbiol Biotechnol 43:1373–1386

122. Li R et al (2014) Expression of recombinant human IL-4 in *Pichia pastoris* and relationship between its glycosylation and biological activity. Protein Expr Purif 96:1–7
123. Yan Y et al (2015) Expression of recombinant chicken IL-5 in *Pichia pastoris* and establishment of indirect ELISA for detection of antibody against the expressed product. Chin Vet Sci/Zhongguo Shouyi Kexue 45:854–860
124. Kamimura D, Ishihara K, Hirano T (2003) IL-6 signal transduction and its physiological roles: the signal orchestration model. Rev Physiol Biochem Pharmacol 149:1–38
125. Fonseca J et al (2009) Interleukin-6 as a key player in systemic inflammation and joint destruction. Autoimmun Rev 8:538–542
126. Li H et al (2011) Large-scale production, purification and bioactivity assay of recombinant human interleukin-6 in the methylotrophic yeast *Pichia pastoris*. FEMS Yeast Res 11:160–167
127. Luo Y et al (2009) Expression, purification, and functional characterization of recombinant human interleukin-7. Protein Expr Purif 63:1–4
128. Li H et al (2009) High level expression and purification of active recombinant human interleukin-8 in *Pichia pastoris*. Protein Expr Purif 68:60–64
129. Zhong Y et al (2014) High-temperature cultivation of recombinant *Pichia pastoris* increases endoplasmic reticulum stress and decreases production of human interleukin-10. Microb Cell Factories 13:1–10
130. Yu K-M et al (2018) Efficient expression and isolation of recombinant human interleukin-11 (rhIL-11) in *Pichia pastoris*. Protein Expr Purif 146:69–77
131. Sun W et al (2016) High level expression and purification of active recombinant human interleukin-15 in *Pichia pastoris*. J Immunol Methods 428:50–57
132. Vijay N et al (2020) Expression of bovine interleukin 15 in *Pichia pastoris* and study on its biological activity: a T-cell activator. Anim Biotechnol 31:357–364
133. Murphy KP Jr et al (1998) Expression of human Interleukin-17 in *Pichia pastoris*: purification and characterization. Protein Expr Purif 12:208–214
134. Zhou L et al (1998) High-level expression of human interleukin-17 in the yeast *Pichia pastoris*. IUBMB Life 46:1109–1116
135. Liu Y et al (2009) Construction and expression of a site-specific mutant of chicken interleukin-18 gene in *Pichia pastoris*. Chin J Prev Vet Med 29:502–506
136. Dong L et al (2009) Expression and analysis of the recombinant human interleukin-21 (rhIL-21) in *Pichia pastoris*. Chin J Biotechnol 25:1711–1717
137. Cai X et al (2010) Expression, purification and characterization of recombinant human interleukin-22 in *Pichia pastoris*. Mol Biol Rep 37:2609–2613
138. Yang J et al (2010) Expression of mice interleukin-23 in *Pichia* and preliminary studies on its biology function. Chin J Microbiol Immunol 26:120–123
139. Liu Y et al (2013) Codon optimization, expression, purification, and functional characterization of recombinant human IL-25 in *Pichia pastoris*. Appl Microbiol Biotechnol 97:10349–10358
140. Ghosalkar A, Sahai V, Srivastava A (2008) Secretory expression of interferon-alpha 2b in recombinant *Pichia pastoris* using three different secretion signals. Protein Expr Purif 60:103–109
141. Salunkhe S et al (2010) Strategies to maximize expression of rightly processed human interferon α2b in *Pichia pastoris*. Protein Expr Purif 71:139–146
142. He H et al (2019) Functional expression of porcine interferon-α using a combinational strategy in *Pichia pastoris* GS115. Enzym Microb Technol 122:55–63
143. Tu Y et al (2016) Extracellular expression and antiviral activity of a bovine interferon-alpha through codon optimization in *Pichia pastoris*. Microbiol Res 191:12–18
144. Gao M-J et al (2012) Methanol/sorbitol co-feeding induction enhanced porcine interferon-α production by *P. pastoris* associated with energy metabolism shift. Bioprocess Biosyst Eng 35:1125–1136

145. Liu PT, Ta TV, Villarete LH (2001) High-yield expression and purification of human interferon α-1 in *Pichia pastoris*. Protein Expr Purif 22:381–387
146. Tian S et al (2013) Construction and characterization of a potent, long-lasting recombinant human serum albumin-interferon α1 fusion protein expressed in *Pichia pastoris*. Protein Expr Purif 90:124–128
147. Shao J et al (2015) Characterization of bovine interferon α1: expression in yeast *Pichia pastoris*, biological activities, and physicochemical characteristics. J Interf Cytokine Res 35: 168–175
148. Huang Y-S et al (2007) Preparation and characterization of a potent, long-lasting recombinant human serum albumin-interferon-α2b fusion protein expressed in *Pichia pastoris*. Eur J Pharm Biopharm 67:301–308
149. Ayed A et al (2008) High level production and purification of human interferon α2b in high cell density culture of *Pichia pastoris*. Enzym Microb Technol 42:173–180
150. Katla S et al (2019) High level extracellular production of recombinant human interferon alpha 2b in glycoengineered *Pichia pastoris*: culture medium optimization, high cell density cultivation and biological characterization. J Appl Microbiol 126:1438–1453
151. Cao R-B et al (2006) Secreted expression of porcine interferon beta in *Pichia pastoris* and its inhibition effect on the replication of pseudorabies virus. Wei Sheng Wu Xue Bao 46:412–417
152. Skoko N et al (2003) Expression and characterization of human interferon-β1 in the methylotrophic yeast *Pichia pastoris*. Biotechnol Appl Biochem 38:257–265
153. Prabhu AA, Veeranki VD, Dsilva SJ (2016) Improving the production of human interferon gamma (hIFN-γ) in *Pichia pastoris* cell factory: an approach of cell level. Process Biochem 51:709–718
154. Prabhu AA et al (2018) Medium optimization for high yield production of extracellular human interferon-γ from *Pichia pastoris*. Korean J Chem Eng 34:1109–1121
155. Prabhu AA, Veeranki VD (2018) Metabolic engineering of *Pichia pastoris* GS115 for enhanced pentose phosphate pathway (PPP) flux toward recombinant human interferon gamma (hIFN-γ) production. Mol Biol Rep 45:961–972
156. Barathiraja S et al (2018) Expression and purification of biologically active bovine interferon λ3 (IL28B) in *Pichia pastoris*. Protein Expr Purif 145:14–18
157. Wang Y et al (2022) Cloning and codon optimization of a novel feline interferon omega gene for production by *Pichia pastoris* and its antiviral efficacy in polyethylene glycol-modified form. Virulence 13:297–309
158. Boue O et al (2000) Antiviral and antiluteolytic activity of recombinant bovine IFN-ω1 obtained from *Pichia pastoris*. J Interf Cytokine Res 20:677–683
159. Josephs SF et al (2018) Unleashing endogenous TNF-alpha as a cancer immunotherapeutic. J Transl Med 16:242
160. Zwyea S, Abdulkareem RA (2020) Cloning, expression and bioactivity of human tumor necrosis factor alpha. Sys Rev Pharm 11:613–621
161. Wang M, Jiang S, Wang Y (2016) Recent advances in the production of recombinant subunit vaccines in *Pichia pastoris*. Bioengineered 7:155–165
162. Wang M et al (2013) Expression, purification, and immunogenic characterization of Epstein–Barr virus recombinant EBNA1 protein in *Pichia pastoris*. Appl Microbiol Biotechnol 97: 6251–6262
163. Kaushik N et al (2016) Casamino acids facilitate the secretion of recombinant dengue virus serotype-3 envelope domain III in *Pichia pastoris*. BMC Biotechnol 16:1–9
164. Shukla R et al (2018) *Pichia pastoris*-expressed bivalent virus-like particulate vaccine induces domain III-focused bivalent neutralizing antibodies without antibody-dependent enhancement *in vivo*. Front Microbiol 8:2644
165. Subathra M et al (2014) Evaluation of antibody response in mice against avian influenza A (H5N1) strain neuraminidase expressed in yeast *Pichia pastoris*. J Biosci 39:443–451

166. Wasilenko JL et al (2010) Cell surface display of highly pathogenic avian influenza virus hemagglutinin on the surface of *Pichia pastoris* cells using α-agglutinin for production of oral vaccines. Biotechnol Prog 26:542–547
167. Athmaram T et al (2013) A simple *Pichia pastoris* fermentation and downstream processing strategy for making recombinant pandemic swine origin influenza a virus hemagglutinin protein. J Ind Microbiol Biotechnol 40:245–255
168. Kwon WT et al (2012) Protective immunity of *Pichia pastoris*-expressed recombinant envelope protein of Japanese encephalitis virus. J Microbiol Biotechnol 22:1580–1587
169. Liang Z, Wang J (2014) EV71 vaccine, an invaluable gift for children. Clin Transl Immunology 3:e28
170. Wang M, Jiang S, Wang Y (2013) Recombinant VP1 protein expressed in *Pichia pastoris* induces protective immune responses against EV71 in mice. Biochem Biophys Res Commun 430:387–393
171. Gurramkonda C et al (2013) Purification of hepatitis B surface antigen virus-like particles from recombinant *Pichia pastoris* and in vivo analysis of their immunogenic properties. J Chromatogr B 940:104–111
172. Limonta-Fernández M, et al (2021) The SARS-CoV-2 receptor-binding domain expressed in *Pichia pastoris* as a candidate vaccine antigen. medRxiv
173. Silva AJD et al (2021) *Pichia pastoris* displaying ZIKV protein epitopes from the Envelope and NS1 induce in vitro immune activation. Vaccine 39:2545–2554
174. Gupta G et al (2016) Immunogenicity of next-generation HPV vaccines in non-human primates: measles-vectored HPV vaccine versus *Pichia pastoris* recombinant protein vaccine. Vaccine 34:4724–4731
175. Zhang C et al (2016) Coxsackievirus A16-like particles produced in *Pichia pastoris* elicit high-titer neutralizing antibodies and confer protection against lethal viral challenge in mice. Antivir Res 129:47–51
176. Celik E et al (2007) Production of recombinant human erythropoietin from *Pichia pastoris* and its structural analysis. J Appl Microbiol 103:2084–2094
177. Nett JH et al (2012) Optimization of erythropoietin production with controlled glycosylation-PEGylated erythropoietin produced in glycoengineered *Pichia pastoris*. J Biotechnol 157: 198–206
178. Zhiyi H et al (2007) Secretion expression and activity assay of a novel fusion protein of thrombopoietin and interleukin-6 in *Pichia pastoris*. J Biochem 142:17–24
179. Wang M et al (2016) Functional expression of human serum albumin-tandem thrombopoietin mimetic peptide fusion protein as a novel thrombopoietin analog in *Pichia pastoris*. Biotechnol Lett 38:779–785
180. Liu DG et al (2007) Functional expression of the globular domain of human adiponectin in *Pichia pastoris*. Biochem Biophys Res Commun 363:769–775
181. Li S et al (2018) A novel purification procedure for recombinant human serum albumin expressed in *Pichia pastoris*. Protein Expr Purif 149:37–42
182. Maity N et al (2022) High level production of stable human serum albumin in *Pichia pastoris* and characterization of the recombinant product. Bioprocess Biosyst Eng 45:409–424
183. Zhu W et al (2021) Medium optimization for high yield production of human serum albumin in *Pichia pastoris* and its efficient purification. Protein Expr Purif 181:105831
184. Adivitiya et al (2016) High level production of active streptokinase in *Pichia pastoris* fed-batch culture. Int J Biol Macromol 83:50–60
185. Ergun BG, Calik P (2016) Lignocellulose degrading extremozymes produced by *Pichia pastoris*: current status and future prospects. Bioprocess Biosyst Eng 39:1–36
186. Varnai A et al (2014) Expression of endoglucanases in *Pichia pastoris* under control of the GAP promoter. Microb Cell Factories 13:57
187. Su X et al (2017) High-level expression and purification of a molluskan endoglucanase from *Ampullaria crossean* in *Pichia pastoris*. Protein Expr Purif 139:8–13

188. Kao MR, Yu SM, Ho TUD (2021) Improvements of the productivity and saccharification efficiency of the cellulolytic beta-glucosidase D2-BGL in *Pichia pastoris* via directed evolution. Biotechnol Biofuels 14:126
189. Jain KK et al (2018) Functional expression of a thermostable endoglucanase from *Thermoascus aurantiacus* RCKK in *Pichia pastoris* X-33 and its characterization. Mol Biotechnol 60:736–748
190. Xia Y, Yang L, Xia L (2018) High-level production of a fungal β-glucosidase with application potentials in the cost-effective production of *Trichoderma reesei* cellulase. Process Biochem 70:55–60
191. Dong C et al (2020) Engineering *Pichia pastoris* with surface-display minicellulosomes for carboxymethyl cellulose hydrolysis and ethanol production. Biotechnol Biofuels 13:108
192. Kiribayeva A et al (2022) Cloning, expression, and characterization of a recombinant xylanase from *Bacillus sonorensis* T6. PLoS One 17:e0265647
193. Xia W et al (2021) Improved production of Streptomyces sp. FA1 xylanase in a dual-plasmid *Pichia pastoris* system. Curr Issues Mol Biol 43:2289–2304
194. Basotra N et al (2018) Expression of catalytically efficient xylanases from thermophilic fungus *Malbranchea cinnamomea* for synergistically enhancing hydrolysis of lignocellulosics. Int J Biol Macromol 108:185–192
195. Bhat SK et al (2022) Cloning and expression of a GH11 xylanase from *Bacillus pumilus* SSP-34 in *Pichia pastoris* GS115: purification and characterization. J Appl Biol Biotechnol 10:1–2
196. Long L et al (2020) Recombinant expression of *Aspergillus niger* GH10 endo-xylanase in *Pichia pastoris* by constructing a double-plasmid co-expression system. J Chem Technol Biotechnol 95:535–543
197. Shang T et al (2017) Enhancement of thermoalkaliphilic xylanase production by *Pichia pastoris* through novel fed-batch strategy in high cell-density fermentation. BMC Biotechnol 17:1–10
198. Gandhi S et al (2015) Expression and characterization of *Geobacillus stearothermophilus* SR74 recombinant alpha-amylase in *Pichia pastoris*. Biomed Res Int 2015:529059
199. Zhoukun L et al (2018) Efficient production and characterization of maltohexaose-forming α-amylase AmyM secreted from the methylotrophic yeast *Pichia pastoris*. Starch 70:1700312
200. Trabelsi S et al (2019) *Aspergillus oryzae* S2 AmyA amylase expression in *Pichia pastoris*: production, purification and novel properties. Mol Biol Rep 46:921–932
201. Wang YC et al (2020) A novel high maltose-forming alpha-amylase from *Rhizomucor miehei* and its application in the food industry. Food Chem 305:125447
202. Wang JR et al (2015) Codon optimization significantly improves the expression level of alpha -amylase gene from *Bacillus licheniformis* in *Pichia pastoris*. Biomed Res Int 2015:248680
203. Parashar D, Satyanarayana T (2017) Production of chimeric acidic alpha-amylase by the recombinant *Pichia pastoris* and its applications. Front Microbiol 8:493
204. Hu X et al (2019) Expression of *Bacillus licheniformis* alpha-amylase in *Pichia pastoris* without antibiotics-resistant gene and effects of glycosylation on the enzymic thermostability. 3 Biotech 9:427
205. Huang M et al (2017) Regulating unfolded protein response activator HAC1p for production of thermostable raw-starch hydrolyzing alpha-amylase in *Pichia pastoris*. Bioprocess Biosyst Eng 40:341–350
206. Wang J et al (2019) High-level expression of *Thermomyces dupontii* thermo-alkaline lipase in *Pichia pastoris* under the control of different promoters. 3 Biotech 9:1–8
207. Sena RO et al (2021) Application of *Rhizomucor miehei* lipase-displaying *Pichia pastoris* whole cell for biodiesel production using agro-industrial residuals as substrate. Int J Biol Macromol 189:734–743
208. Tian M et al (2021) Crude glycerol impurities improve *Rhizomucor miehei* lipase production by *Pichia pastoris*. Prep Biochem Biotechnol 51:860–870

209. Zhang X-F et al (2019) High-level expression of *Aspergillus niger* lipase in *Pichia pastoris*: characterization and gastric digestion *in vitro*. Food Chem 274:305–313
210. Abu ML et al (2021) The use of response surface methodology for enhanced production of a thermostable bacterial lipase in a novel yeast system. Prep Biochem Biotechnol 51:350–360
211. de Macedo RJ et al (2019) Continuous operation, a realistic alternative to fed-batch fermentation for the production of recombinant lipase B from *Candida antarctica* under the constitutive promoter PGK in *Pichia pastoris*. Biochem Eng J 147:39–47
212. Zhou Q et al (2019) High-level production of a thermostable mutant of *Yarrowia lipolytica* lipase 2 in *Pichia pastoris*. Int J Mol Sci 21:279
213. Xie Z, Fong WP, Tsang PW (2020) Engineering and optimization of phosphate-responsive phytase expression in *Pichia pastoris* yeast for phytate hydrolysis. Enzym Microb Technol 137:109533
214. Ranjan B, Satyanarayana T (2016) Recombinant HAP phytase of the thermophilic mold *Sporotrichum thermophile*: expression of the codon-optimized phytase gene in *Pichia pastoris* and applications. Mol Biotechnol 58:137–147
215. Parashar D, Satyanarayana T (2016) Enhancing the production of recombinant acidic α-amylase and phytase in *Pichia pastoris* under dual promoters [constitutive (GAP) and inducible (AOX)] in mixed fed batch high cell density cultivation. Process Biochem 51: 1315–1322
216. Yang M et al (2015) Extracellular expression of alkaline phytase in *Pichia pastoris*: influence of signal peptides, promoters and growth medium. Biotechnol Rep (Amst) 6:112–118
217. Helian Y et al (2020) A multistrategy approach for improving the expression of *E. coli* phytase in *Pichia pastoris*. J Ind Microbiol Biotechnol 47:1161–1172
218. Li C et al (2015) Combined strategies for improving expression of *Citrobacter amalonaticus* phytase in *Pichia pastoris*. BMC Biotechnol 15:88
219. Xu G et al (2019) Expression of a thermo- and alkaliphilic fungal laccase in *Pichia pastoris* and its application. Protein Expr Purif 154:16–24
220. Bronikowski A et al (2017) Expression of a new laccase from *Moniliophthora roreri* at high levels in *Pichia pastoris* and its potential application in micropollutant degradation. AMB Express 7:73
221. Liu H et al (2015) Overexpression of a novel thermostable and chloride-tolerant laccase from *Thermus thermophilus* SG0.5JP17-16 in *Pichia pastoris* and its application in synthetic dye decolorization. PLoS One 10:e0119833
222. Wang J, Lu L, Feng F (2017) Combined strategies for improving production of a thermo-alkali stable laccase in *Pichia pastoris*. Electron J Biotechnol 28:7–13
223. Yang H et al (2016) High-level expression of Proteinase K from *Tritirachium album Limber* in *Pichia pastoris* using multi-copy expression strains. Protein Expr Purif 122:38–44
224. Mechri S et al (2021) Cloning and heterologous expression of subtilisin SAPN, a serine alkaline protease from *Melghiribacillus thermohalophilus* Nari2A in *Escherichia coli* and *Pichia pastoris*. Process Biochem 105:27–41
225. Pereira WES et al (2020) A collagenolytic Aspartic Protease from *Thermomucor indicae-seudaticae* expressed in *Escherichia coli* and *Pichia pastoris*. Appl Biochem Biotechnol 191: 1258–1270
226. Ma X et al (2016) Expression, purification and identification of a thermolysin-like protease, neutral protease I, from *Aspergillus oryzae* with the *Pichia pastoris* expression system. Protein Expr Purif 128:52–59
227. Sun Q et al (2018) A novel aspartic protease from *Rhizomucor miehei* expressed in *Pichia pastoris* and its application on meat tenderization and preparation of turtle peptides. Food Chem 245:570–577
228. Karaoglan M, Erden-Karaoglan F (2020) Effect of codon optimization and promoter choice on recombinant endo-polygalacturonase production in *Pichia pastoris*. Enzym Microb Technol 139:109589

229. Abdulrachman D et al (2017) Heterologous expression of *Aspergillus aculeatus* endo-polygalacturonase in *Pichia pastoris* by high cell density fermentation and its application in textile scouring. BMC Biotechnol 17:15
230. Baghban R et al (2020) Mutational analysis of ocriplasmin to reduce proteolytic and autolytic activity in *Pichia pastoris*. Biol Proced Online 22:25
231. Neffendorf JE et al (2017) Ocriplasmin for symptomatic vitreomacular adhesion. Cochrane Database Syst Rev 10:CD011874
232. Prospero Ponce CM et al (2016) Ocriplasmin: who is the best candidate? Clin Ophthalmol 10: 485–495
233. Craig TJ et al (2015) Characterization of anaphylaxis after ecallantide treatment of hereditary angioedema attacks. J Allergy Clin Immunol Pract 3:206–212.e204
234. Duffey H, Firszt R (2015) Management of acute attacks of hereditary angioedema: role of ecallantide. J Blood Med 6:115–123
235. Thompson CA (2010) FDA approves kallikrein inhibitor to treat hereditary angioedema. Am J Health Syst Pharm 67:93
236. Hoy SM (2020) MYL1501D insulin glargine: a review in diabetes mellitus. BioDrugs 34:245–251
237. Orders M (2021) In brief: Semglee – a new insulin glargine for diabetes. Med Lett Drugs Ther 63:14–15
238. Lavalle-Gonzalez FJ, Khatami H (2014) The biosimilar insulin landscape: current developments. Postgrad Med 126:81–92
239. Peters AL et al (2015) Biosimilars and new insulin versions. Endocr Pract 21:1387–1394
240. Abraham P et al (1999) Evaluation of a new recombinant DNA hepatitis B vaccine (Shanvac-B). Vaccine 17:1125–1129
241. Chakma J et al (2011) Indian vaccine innovation: the case of Shantha Biotechnics. Glob Health 7:9
242. Velu V et al (2007) Comparison of three different recombinant hepatitis B vaccines: GeneVac-B, Engerix B and Shanvac B in high risk infants born to HBsAg positive mothers in India. World J Gastroenterol 13:3084–3089
243. Balan S et al (2007) Site-specific PEGylation of protein disulfide bonds using a three-carbon bridge. Bioconjug Chem 18:61–76
244. Wang Y et al (2019) The application of HPLC/MS analysis with a multi-enzyme digest strategy to characterize different interferon product variants produced from *Pichia pastoris*. Amino Acids 51:1353–1363

Agro-industrial Residues: An Eco-friendly and Inexpensive Substrate for Fungi in the Development of White Biotechnology

19

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Abstract

The population growth in the twenty-first century, constant exploitation of natural nonrenewable resources, and industrial pollution have forced humanity to look for alternative renewable resources, and so far the agro-industrial residues are one of the most suitable alternatives. Being rich in carbohydrates (cellulose and hemicellulose) and novel biochemical compounds (polyphenolics), agro-residues can be utilized by agricultural, biotechnological, pharmaceutical, and manufacturing industries. Being available abundantly in nature, they are cost-effective, renewable, low carbon-emitting, and eco-friendly in nature. These features make their way to bio-based refinery, and it is also one of the best ways toward green technologies and agro-waste utilization for the generation of value-added products. This chapter describes the wide range of agro-residues available and their application in the context of fungal white biotechnology.

Keywords

Agro-industrial residue · Renewable resources · Fungal white biotechnology · Polyphenols · Sugarcane bagasse · Biorefinery · Biofuel

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19.1 Introduction

The rapid expansion of population and the constant growth of global economy have increased energy demand, and nearly 80% of this demand is met by using nonrenewable energy resources [1]. Fossil fuel consumption has resulted in greenhouse gas emissions, which have drastic effects on the climate. They are limited in nature and may even deplete in the near future [2]. Lignocellulosic biomasses are regarded as an effective and sustainable solution to this problem as they can fulfill the energy demand as well as reduce net carbon emission. Lignocellulosic biomass (LCB) is abundantly available in nature. LCB is a highly renewable, economical, and eco-friendly feedstock containing sugar polymers along with organic moieties such as lignin and suberin that can be processed to generate value-added products such as second-generation biofuels, organic acids, animal feeds, and bio-sourced compounds [3].

LCB has a complex three-dimensional structure, in which cellulose fibers are enveloped by the condensed structure formed by hemicellulose and lignin (Fig. 19.1). Cellulose is a homopolymer of glucose molecules which are linked by β-1,4-glycosidic linkage whereas hemicellulose is a heteropolymer which consists of a variety of sugars such as xylose, mannose, arabinose, and galactose. Lignin is composed of polyphenolic compounds (coniferyl alcohol, sinapyl alcohol, and coumaryl alcohol) that act as cementing material between cellulose fibers [4]. LCB can be categorized into biomass, virgin biomass, and energy crops. Trees, bushes,

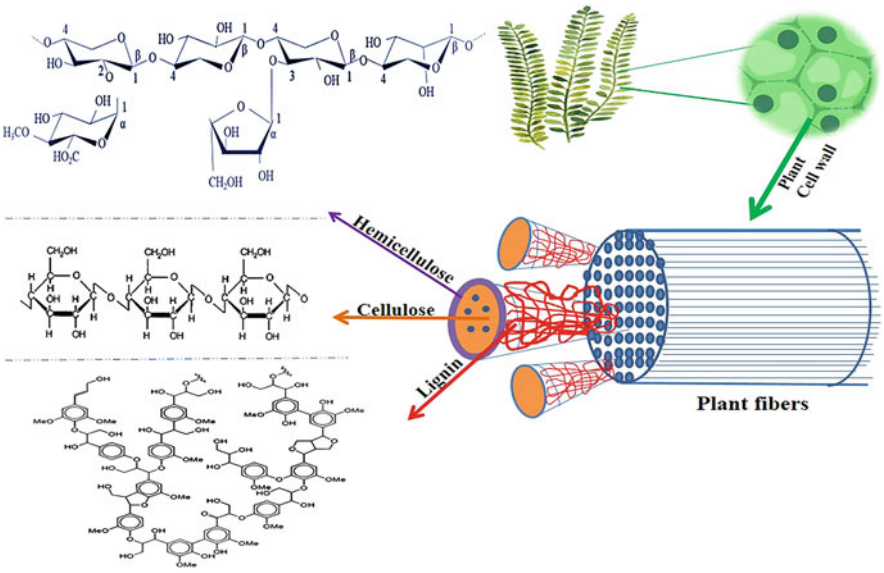

Fig. 19.1 Schematic representation of lignocellulosic biomass composition

and sand grasses are examples of virgin biomass, whereas agricultural residue, stover, and bagasse are examples of waste biomass. Energy crops are raw materials grown specifically for the production of second-generation biofuels as they offer high biomass to fuel conversion [5]. The conversion of LCB for the production of biofuels and energy is based on two main approaches. The use of biochemical processes involving enzymatic and microbial conversion is employed when LCB substrate has C/N ratio below 30 and humidity content above 30%. For the LCB substrates having C/N ratio higher than 30 and humidity content lower than 30%, thermochemical processes can be utilized. In recent years, attempts have been made to produce a wide range of new-generation biofuels such as biohydrogen, ethanol, butanol, dimethylfuran, levulinic acid, and gamma-valerolactone from LCB [6].

LCB is considered a good sustainable resource because of its abundant availability, renewability, low cost, and global mass production. Apart from biofuels, LCBs are used in wood processing industries, paper and pulp industries, biotechnological industries as well as nutrient-rich animal feed production. According to Qaisar et al. [7], the annual global production of LCBs was more than 180 billion tons and most of it remains unutilized. A small fraction of unutilized LCBs has been used as fodder for animals [8] or dumped in landfills. However, due to high transportation costs, the major part is incinerated on-site, which leads to severe problems of greenhouse emission and high particulate matter in the air. Therefore, utilization of LCBs for white biotechnological purposes will be a truly holistic and green approach, as it will not only provide the substrate for the production of value-added products but also reduce the problem of solid waste disposal land and air pollution.

19.2 Types of Agro-industrial Residues

In recent years, there has been a growing tendency toward more effective use of agro-industrial residues such as cassava bagasse, sugarcane bagasse, sugar beet pulp, coffee pulp/husk, apple pomace, and so on.

19.2.1 Sugarcane Bagasse

Saccharum officinarum is the scientific name for sugarcane, which belongs to the Gramineae family. It may be found in tropical and subtropical regions all over the world. It may reach a height of 8–20 feet and is roughly 2 in. thick. There are several distinct horticultural types, each with its unique stem color and length. About 200 nations grow sugarcane, and Brazil is the world's largest producer, accounting for roughly 25% of global output. India, Pakistan, China, and Thailand are the next major producers. India is the world's second-largest sugarcane producer. Sugarcane is commonly used to make falernum, sugar, rum, soda, molasses, and ethanol for transportation. Bagasse has a low ash content, which makes it ideal for use in bioconversion processes involving microbial cultures. Bagasse may also be regarded a rich solar energy reservoir in comparison to other agricultural residues due to its

high yields (about 80 tons/ha vs. 1 ton/ha for wheat, 2 tons/ha for other grasses, and 20 tons/ha for trees, respectively) and yearly regeneration ability [9].

Sugarcane bagasse is very rich in cellulose and hemicellulose content, i.e., 50% and 25%, respectively. It also contains about 25% of lignin as well as minimum ash content. To be more specific, there is about 50% α-cellulose and 30% pentosans, with 2.4% ash content [9]. Because of having very high amount of cellulose and hemicellulose, sugarcane bagasse is considered one of the most promising sources of carbon and energy. Sugarcane bagasse, a rich source of energy and carbon, is widely utilized as raw material for the production of various biotechnologically significant products such as organic acids [10], enzymes [11], animal feed [12], mushrooms [13], bioplastics [14] as well as biofuels [15]. Moreover, it is also used for the generation of electricity and paper production.

19.2.2 Cassava Bagasse

Cassava (*Manihot esculenta* Crantz) is a short-lived perennial that grows 1– 5 meters tall and belongs to the Euphorbiaceae family. Cassava is originated in South America, most likely in eastern Brazil. It is a bushy plant with aerial and subterranean portions that produce tubers. With a trunk and branches, the height of the aerial section can reach up to 4 meters. The subterranean portion is composed of two types of roots: those that provide nourishment to the plant and those that are arranged axially around the stem. These are known as tubers and are the plant's edible portions. After rice and corn, the tropical root crop cassava (*Manihot esculenta* Crantz) is the third most important source of calories in the tropics [16].

Industrially, cassava tuber processing is mostly done to extract flour and starch, which results in more liquid and solid residues (processing for flour produces solid residues, while processing for starch produces liquid residues) [17]. Brown peel, inner peel, useless roots, bagasse, and flour trash are examples of solid residues, with bagasse being the most common solid residue. Apart from this, cassava bagasse has also been used for the preparation of nanofibers [18].

19.2.3 Oil Cakes

Oil cakes are solid remnants leftover after expulsion or solvent extraction of oil from a plant component like a seed. Edible oil cakes are made from edible oil-bearing seeds that are utilized to cover a portion of the nutritional needs of either animal feed or human consumption. On the other hand, nonedible oil cakes are made from seeds that do not contain poisonous chemicals or other contaminants [19]. The chemical composition of different oil cakes are described in Table 19.1.

Nonedible oil cakes, made from neem, castor, mahua, and karanja, are commonly utilized as manures. Soybean cake, rapeseed cake, cottonseed cake, groundnut cake, sunflower cake, copra cake, and linseed cake are the most popular edible oil cakes in the world [21]. Soybean cake accounts for 54% of the overall production volume of

Table 19.1 Chemical composition of major oil cakes (adapted from [20])

Oil cakes	Dry matter (%)	Carbohydrate (%)	Crude protein (%)	Fat (%)	Crude fiber (%)	Ash (%)
Sunflower	93	23.0	35.6	1.68	28.41	7.36
Copra	89.9	42.4	20.9	8.0	11.5	5.5
Sesame	93.9	21.0	48.2	2.3	6.4	12.6
Soybean	90.3	23.6	51.8	0.9	17.8	7.3
Rapeseed	90.75	32.2	42.8	4.1	12.1	7
Olive oil	72.8	10.1	4.77	8.72	49.14	2.36
Linseed	88.9	36.0	33.2	2.8	8.1	5.4
Groundnut	90	14.1	45.6	2.47	8.3	5.02
Safflower	93.1	20.1	44	5.9	12.1	7.2
Palm kernel	93	45.5	17.5	7.4	11.9	4.8
Cottonseed	91.53	27.0	41.5	5.75	14.67	6.46

the different types of oil cakes mentioned above, with rapeseed cake accounting for 10% and cottonseed cake accounting for 10%.

Oil cakes have major potential for the production of various industrially significant products. Various microbial enzymes including amylase [22], lipase [23], xylanase [24], tannase [25], protease [26], and phytase [27] have been produced using a single type of oil cakes or with a combination of different oil cakes, as a raw agro-industrial residue. Oil cakes have been used not only as raw materials but also as media supplements, by extracting them in the form of soy peptone trypticase soy agar, soy protein isolate or concentrate as well as soy flour, which supplements the needs of nitrogen sources in the culture media [28]. Moreover, oil cakes have also been used for the production of secondary metabolites. Soybean cake and cottonseed cake were used as nitrogen sources for the production of antibiotics [29]. In addition, oil cakes have also been explored for the production of secondary metabolites such as biosurfactants, which have potential applications in bioremediation, microbial enhanced oil recovery (MEOR), and food processing industries [30].

19.2.4 Cereal Straw and Bran Residues

Cereal straw is one of the world's most abundant and renewable lignocellulosic waste materials with a great potential in white biotechnology. The estimated global yield of cereal straw is 2.9 billion tons per annum [31], which directly displays the abundance of agricultural waste biomass available to be utilized as a renewable resource. There are various crops such as wheat, barley, rice, oats, and rye, which are incorporated under the title "cereals." The nonedible parts of these crops, straw and bran, are important agro-residues. Cereal straw is made up of a high percentage of biological macromolecules such as lignin, cellulose, and hemicellulose. Both cellulose and hemicellulose are polymers of sugar monomers linked via glycosidic

Table 19.2 Chemical composition of various agro-residues

Agro-industrial substrate	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Cellulose/ lignin	References
Wheat straw	31.5–39.5	21.2–29.0	5.6–15.0	2.2–5.3	[33]
Rice straw	22.8–38.4	17.7–28.5	6.4–18.0	3.6–5.9	[34]
Rice husk	28.0–43.0	17.5–20.6	21.5–22.5	1.3–1.9	[35]
Corn stover/husk	36.4–40.0	25.0–29.0	13.0–21.0	2.1–2.3	[36]
Corn cobs	28.0–45.0	35.0–43.0	11.0–17.0	2.5–2.7	[37]
Cotton hulls	52.0–90.0	5.0–20.0	4.0–12.0	5.0–11.2	[38]
Sugarcane bagasse	26.6–40.0	19.0–30.0	19.0–23.3	1.4–2.2	[39]
Soft wood chips/ sawdust	37.7–49.5	10.7–25.0	26.1–29.5	1.4–1.7	[40]
Hard wood chips/ sawdust	42.9–45.1	22.0–33.0	24.0–26.0	1.7–2.0	[41]
Coffee pulp/husk	23.0–29.1	15.1–17.1	13.0–26.0	0.89–0.94	[42]

linkages. Upon hydrolysis, some sugars can be fermented into various products such as acetic acid, levulinic acid, ethanol, and acetone. Moreover, these sugars can also be used for the fermentation of antibiotics and enzymes [32].

Chemically, agro-residues are mainly composed of cellulose, hemicellulose, and lignin, where their proportion varies with different types of residual substrates (Table 19.2).

Cereal straws such as rice straw, wheat straw, barley straw, oats straw, and rye straw as well as bran residues are one of the most abundant agricultural residues in the world. For long, humans have used these residues for various purposes.

Since ancient times, people are used to burning the residual biomass on the fields and also utilizing as a firewood fuel to generate energy as the direct combustion is easy and economic. However, the incomplete combustion imposes serious environmental pollution problems due to low combustion efficiency. Recently, researchers have been working on improving calorific values and combustive efficiency of various straw and bran residues [43], which may enhance the usage of straw for the production of industrially significant products. Moreover, the agro-waste and fibrous lignocellulosic materials have been intensively utilized in paper and pulp industries since the third century BC [44]. Paper and pulp industries are always interested in the cellulosic portion of the lignocellulosic biomass. Thus, the remaining part containing lignin and hemicellulose is almost wasted in the form of black liquor. This black liquor also contains strong acids and alkali, which not only cause severe water pollution problems but also increase the cost of the process. Thus, researchers are involved in the development of some novel biotechnological processes that may be cost-effective as well as eco-friendly. Such biotechnological processes may involve the application of fungal enzymes, organic acids, and biosurfactants.

In addition, cereal straws and bran residues have also been used as animal feed. Some countries such as Australia and New Zealand have enough grasslands which can afford forage-based animal husbandry whereas other developed countries such

as Switzerland and the United States can meet the expense for grain-based animal husbandry. In contrast, countries such as India and China have high population-to-land ratio; thus they cannot afford forage or grains for their fast-growing animal husbandry [45, 46]. Therefore, abundant straw and bran residues can be considered as sustainable resources for animal feed. However, the direct usage of such residues as animal feed is limited due to their complex composition having higher amounts of lignin and lower amounts of proteins, which results in poor digestibility and, as a result, lower nourishment [47]. It is reported that ruminants such as sheep and cattle can only digest about 50% of the ingested straw; on the other hand, pig can digest maximally up to 25% [48]. Thus nowadays it has become necessary to improve the digestibility and protein content of straw residues using various straw processing technologies such as silage and straw ammonization.

19.2.5 Banana Pseudostem

Banana is one of the tallest monocotyledons herbaceous plants [49]. The banana plant belongs to the Musaceous family. Banana is the oldest and most economically significant cultivated crop in the world. The banana plant is also known as a cash or staple crop. Bananas originated from Southeastern Asia and the Western Pacific region [50]. Banana plants are also referred to as a "Kalpathru," a plant of virtues [51]. The meaning of banana is a finger, and the word comes from Arabic "Banaana" or "Banaan" which means finger tiles [52]. The banana plant is grown all over the world. Banana is the fourth largest cultivated fruit in the world after grapes, citrus, and apple fruit [53]. India, China, Philippines, and Brazil are the maximum banana-producing countries. In 2020, the production of banana fruit in the world reached 20 million tons. India produced 32 million metric tons of banana in 2020. India contributes 26% of the world's banana production.

Banana pseudostem is made up of tightly packed, overlapped twisting leaf sheaths with a central core and because of this, the stem of banana is called "Pseudostem" [54]. Banana plants produce only one bunch of bananas during the life cycle. After harvesting the bunch of bananas, the residues (banana pseudostem) are left on the plantation as a waste. It generates disposal and environmental problems [55]. Such problems can be reduced by using banana pseudostem in the preparation of value-added products. Banana pseudostem is also used in solid-state fermentation as a solid material.

Each part of the banana pseudostem is useful in the preparation of value-added products. Li et al. [56] reported that the banana pseudostem is a good source of holocellulose and low amount of lignin which make the banana pseudostem an excellent source for pulping and papermaking. Sap, fibers, central core, and sutures are four parts of banana pseudostem that can be utilized in order to prepare value-added products such as mordants, liquid fertilizers, microcrystalline cellulose, fabric, yarn, candy, pickles as well as compost.

Banana pseudostem sap is an extract of banana pseudostem which is brown in color. Banana pseudostem is known for its medicinal and industrial applications.

Thorat and Bobade [55] reported that the juice of the banana pseudostem is beneficial in dissolving the stone in the kidney and urinary bladder. The sap of the banana pseudostem is also effective against the jaundice. In addition, sap has also been used as a natural mordant in the dying processes [57].

Banana pseudostem is made up of 14-18 leaf sheaths which are mainly divided into three parts, i.e., outer sheath, intermediate sheath, and central core. The fibers of the outer sheaths are very brittle and can be easily broken down. The central core is a pulpy matter which is not useful for fiber extraction. Thus only the intermediate sheaths are used for the production of fibers. The fibers can be extracted either mechanically, chemically, or enzymatically using fungal enzymes such as laccases, pectinases, and cellulases. This banana fiber has many applications such as in the preparation of yarns, handicrafts, handbags, ropes, purses, carpets, tissue papers, microcrystalline cellulose (MCC), and doormats [52, 58]. Many researchers reported that the banana pseudostem fibers are rich in cellulosic content [54, 59]. In particular MCC can be prepared by treating α-cellulose by using chemical or biological or physical treatment [60]. MCC has an excellent property in pharmaceutical, food, and cosmetic industries. MCC is used in pharmaceutical industries as a binder or adsorbent, in food industries as a stabilizer, thickener, emulsifier, anticaking or bulking agent due to the excellent binding property, and in polymer composite as a mechanical reinforcing agent [61].

19.3 Lignocellulosic Biomass-Based Biorefinery: A Circular Economy Approach

A circular economy is "a model of production and consumption, which involves sharing, leasing, reusing, repairing, refurbishing and recycling existing materials and products as long as possible" [62]. Fungal biotechnology has the ability to utilize the agro-industrial waste and the ability for sustainable production of irrepressible sources of feed, food, fuels, chemicals, construction materials, textiles, automotive and transportation industries and beyond. As well as it can advance the transition from petroleum-based economy into a bio-based circular economy [63].

Biorefinery is an emerging concept which is an amalgamation of biomass conversion processes and industrial facility for the production of producing fuels, power, chemicals, and a variety of other value-added products from biomass, using a wide range of technologies (Fig. 19.2) [5]. Consequently, the concept of biorefinery is considered analogous to that of petroleum refinery with the main difference of using renewable plant-derived materials instead of nonrenewable fossil-derived petroleum crude [64, 65]. The wide range of technologies employed in biorefinery can provide bio-based products such as biomaterials (fibers and pulp for paper industries, composite materials, hydrogels), biofuels (ethanol, biodiesel, butanol, and methane), and a variety of biochemical compounds (levulinic acid, acetic acid, polysaccharides, etc.) through fractionation, fermentation, and purification processes. Therefore agro-industrial residues have a wide range of application as

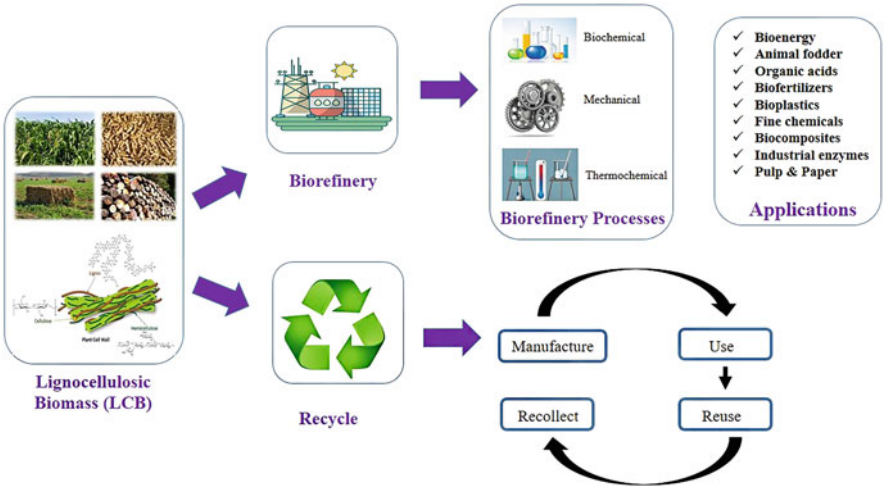

Fig. 19.2 Bio-based refinery processes and their applications

an alternative to nonrenewable resources which makes it a valuable commodity around the globe [66].

19.4 Major Applications of Agro-industrial Residues

19.4.1 Agro-residues in Biotechnologically Significant Enzyme Production

Agro-residues are a rich source of nutrients which can be utilized to support the growth and development of microbes for the purpose of production of various enzymes such as laccase, xylanase, cellulase, and xylosidases[67]. Table 19.3 is a compilation of enzymes produced from various agro-industrial wastes.

19.4.1.1 Laccase

Laccases produced by fungi are very crucial and more important at the industrial level. Laccases have wide applications in various fields such as textile, pharmaceutical and chemical industries, food industry, wood processing, and many more. Laccases (E.C. 1.10.3.2) belong to the class oxidoreductase, which has the ability to oxidize diphenols as well as can use the molecular oxygen as electron acceptor. Laccases belong to a category of polyphenol oxidases which contain copper atom in the catalytic center and therefore are called multicopper oxidases [81]. Fungal genera belonging to Ascomycetes, Deuteromycetes as well as Basidiomycetes have been reported for their laccase production capabilities.

Rebhun et al. [82] reported laccase production using agro-industrial waste with the help of white-rot basidiomycetes such as *Cerrena unicolor* and *Cucurbita*

Table 19.3 Production of industrially significant enzymes from LCB using fungi

Agro-industrial substrate	Fungal species	Enzymes	References
Wheat straw, rice straw, wheat bran, rice bran	*Fusarium incarnatum* UC-14, *Trametes versicolor, Phanerochaete chrysosporium, Pleurotus ostreatus* HP-1	Laccase	[68–71]
Finger millet, bajra, barnyard millet, paddy, maize stover, jowar, foxtail millet, proso millet straw	*Aspergillus flavus Ganoderma lucidum* IBL-05 *Phanerochaete chrysosporium*	Lignin peroxidase	[72, 73]
Wheat, straw, oil palm empty fruit bunches	*Aspergillus tubingensis* JP-1 *Aspergillus sp.* LPB-5	Xylanase	[74, 75]
Wheat bran, textile wastes consisting of cotton and polyester	*Trichoderma reesei* NCIM 1186 *Penicillium citrinum* NCIM 768 *Aspergillus niger* CKB	Cellulase	[76, 77]
Wheat bran, mosambi peel, mosambi bagasse, lemon peel, rice bran, banana peel, sugarcane bagasse	*Aspergillus oryzae*	Pectinase	[78]
Oil palm cake	*Pseudolagarobasidium sp.* PP17-33	Manganese peroxidase	[79]
Lime, grape, tangerine, and sweet orange peels	*Penicillium italicum* and *Trichosporonoides oedocephalis*	Mannanase, xylanase	[80]
Barley bagasse	*Trichoderma koningiopsis 2OI2A1M*	β-xylosidases, xylanase, β-glucosidases	[17]

maxima and conclude that wheat bran was the excellent substrate for growth and for fermentation by *C. unicolor*, enabling a better production of laccase (87.450 IU/L on day 7). Freixo et al. [83] utilized tomato pomace as the only carbon source and reported the maximum laccase production after the third day (362 U/L of fermentation broth). Birhanli et al. [84] utilized lignocellulosic wastes such as sunflower receptacle, apricot seed shell, and bulrush for laccase production under semisolid-state and submerged fermentation (SMF) conditions from *Trametes trogii* (Berk.). Wang et al. [85] reported the increased laccase production by *Trametes versicolor* using corn steep liquor as both nitrogen source and inducer. At present the focus has been on using a mixture of agro-residues, instead of individual ones. An et al. [86] reported that laccase production in *P. ostreatus* was improved after inclusion of cottonseed hulls with corncob and straw. In a recent study involving the use of *Lentinus strigosus* isolated from Amazon, a total of 176.23 U/mL laccase activity

was reported after 6 days and as a substrate mixed lignocellulosic biomass was used, which is composed of cellulose (19.16%), hemicellulose (32.83%), and lignin (6.06%) [87].

19.4.1.2 Cellulase

Cellulose is one of the most plentiful carbohydrates in plants. Structurally, cellulose is a linear biopolymer of hydroglucose units linked by the β-1,4-glycosidic linkages. The enzyme employed for cellulose hydrolysis is cellulase (E.C. 3.2.1.4), which decomposes cellulose into shorter oligomeric chains like cellodextrin, cellobiose, and monomeric sugar units like glucose. Depending on the structure and function, cellulases can be categorized into three categories, viz. endoglucanases, exoglucanases or cellobiohydrolases, and β-glucosidases or cellobiases. In spite of being classified into different categories, these enzymes work collaboratively and in a coordinated manner to catalyze the hydrolysis of the complex cellulose. According to the classical hydrolysis theory, endoglucanases randomly hydrolyze the cellulose chains along the amorphous regions by a mechanism of adsorption and desorption and in turn produce cellodextrin. On the other hand, cellobiohydrolases gradually hydrolyze the crystalline cellulose regions from either the reducing or nonreducing end that liberate cellobiose as their main product whereas β-glucosidases hydrolyze the released soluble cello-oligomers to monomeric glucose units [88].

Picart et al. [89] using rice straw produced cellulase by *Penicillium* sp. And the enzyme reported was stable at temperature 65 °C and pH 4–5. Waghmare et al. [90] compare various agriculture wastes like sorghum husks, grass powder, corn straw, paddy straw, sugarcane bagasse, and sugarcane barbojo for cellulolytic enzyme production; the best carbon sources for enzyme production was grass powder and sugarcane barbojo. Olajuyigbe et al. [91] reported the production of thermostable crude cellulase enzymes on corncob by *Sporothrix carnis*. The highest production of enzyme achieved was at 96 h with 2.5% inoculum, activity (285.7 U/mL), pH 6.0, and temperature 80 °C. Perez et al. [92] isolated thermophilic fungi *Myceliophthora thermophila* and reported 18.75 U g^{-1} d^{-1} cellulase activity using sugarcane bagasse and wheat bran as a substrate. Rayhane et al. [93] utilized a mixture of lignocellulosic residue consisting of vine shoots, jatropha cake, olive pomace, and olive oil to produce cellulase using *Trichoderma asperellum*. In one of the important studies recently, Laothanachareon et al. [94] investigated the effect of various agro-residues on cellulase production by using 23 different strains of *Aspergillus niger* and concluded that CMCase and β-glucanase activity as only appeared when carbon source was switched from basal sugar medium to agro-residue biomass (sugarcane bagasse).

19.4.1.3 Hemicellulases

Hemicellulases are a group of enzymes that break down hemicellulose, which is a major component of plant cell walls. Hemicellulases target different types of hemicelluloses such as pentosans, xylans, galactans, mannans, and glucans. Hemicellulases are equipped with functional modules that are capable of digesting glycosidic bonds as well as esterified side chain groups. Acetyl and feruloyl esterases

hydrolyze acetate or ferulic acid side groups in the plant cell wall structure. Some of the most common hemicellulases that act upon glycosidic bonds include α-glucuronidases, α-arabinofuranosidases, α-d-galactosidases, and mannanases.

Among hemicellulases, xylanase (EC 3.2.1.8) is a class of enzymes that degrade the linear polysaccharide xylan into xylose, thus breaking down hemicellulose, one of the major components of plant cell walls. Xylans principally consist of D-xylose as its monomeric unit and traces of L-arabinose [95]. The use of easily available and cost-effective agriculture residues such as wheat bran, corncobs, and wheat straw provides suitable methods to achieve higher xylanase yields. Gawande et al. [96] evaluated many lignocellulosic residues such as wheat bran, sugarcane bagasse, rice straw, and soya bean hulls for xylanase production from *A. terreus* and *A. niger*. Highest xylanase production was observed in wheat bran. Milagres et al. [97] showed the production of high level of thermostable xylanase (1597 U/g xylanase activity) after 10 days of SSF from *Thermoascus aurantiacus*. Patel and Prajapati [98] reported wheat husk, rice bran, and rice straw as efficient agro-residues for xylanase production by using *Cladosporium sp.,* and the maximum xylanase activity was found with rice bran. Cunha et al. [99] optimized the production of xylanase by *Aspergillus foetidus* using soybean residues and achieved 13.98 U/mL enzyme activity. Most recently Ismail et al. [100] isolated *Aspergillus flavus* AW1 which was able to produce xylanase using corn cobs as a substrate.

19.4.1.4 Pectinase

Pectinases are a group of enzymes that break down pectin, a polysaccharide found in plant cell walls, through hydrolysis, transelimination, and deesterification reactions. Among the agro-residues fruit waste and peels are a rich source of pectins and potentially can be used for the production of pectinase. Rangarajan et al. [101] suggested orange peel as an important agro-residue that can be used to produce pectinase and reported exopectinase activity of 6800 IU/g with orange peel extract using *Aspergillus niger*. Satapathy et al. [102] were able to produce 1366.30 U/mL pectinase using *Aspergillus parvisclerotigenus* KX928754 in liquid static surface fermentation, 973.12 U/mL with sugarcane bagasse, and 686.7 U/mL with spent tea residues.

19.4.2 Agro-industrial Residues in Mushroom Cultivation

A large quantity of agricultural crop residues in terms of lignocellulosic waste as well as organic content rich agro-industrial by-products is generated annually, which are worth getting recovered and transformed into value-added products. These agro-residues have been utilized for the production of industrially significant organic acids, biosurfactants, enzymes, biofertilizer or biopesticide, flavors, ethanol, bioactive secondary metabolites, animal and aquaculture feedstocks, therapeutic compounds as well as edible and medicinal mushrooms, and also for bioremediation of hazardous compounds, biological detoxification of agro-industrial residues, and

Countries	Mushroom Production (Tons)				
	1998	2003	2008	2013	2018
Africa	9,874	12,481	16,113	22,745	28,767
Australia	38,895	39,288	46,808	49,954	51,222
Canada	72,880	87,937	79,990	1,32,448	1,38,412
France	1,98,934	1,69,770	1,38,783	1,04,461	83,013
Germany	60,000	50,000	57,000	59,884	73,231
India	13,500	40,000	37,000	17,000	60,733
Ireland	62,000	69,000	55,500	67,700	65,300
Italy	59,343	96,090	3,42,000	60,453	70,673
Japan	74,217	65,400	67,500	65,966	65,747
Netherlands	2,46,000	2,63,000	2,55,000	3,23,000	3,00,000
New Zealand	8,400	8,500	7,723	2,129	2,906
Poland	1,03,214	1,34,873	1,85,000	2,49,148	2,80,232
Republic of Korea	16,000	24,957	28,361	19,742	22,737
Spain	80,000	1,29,205	1,33,548	1,49,700	1,66,250
UK	1,09,500	81,000	70,200	85,484	98,500
USA	3,84,540	3,87,601	3,68,591	4,08,157	4,16,050
Viet Nam	12,000	16,640	19,085	21,000	23,659

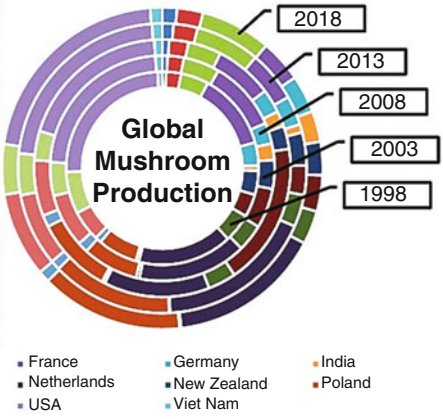

Fig. 19.3 Worldwide mushroom production (Data source: FAOSTATS—Food and Agriculture Organization [111])

biopulping under solid-state fermentation (SSF) with the help of fungi from Ascomycetes and Basidiomycetes [103–107].

Cultivation of mushrooms is one of the prominent biotechnological processes for agro-waste valorization. Mushroom cultivation is an economically feasible and widely performed SSF process in which nutrients available in lignocellulosic material are transformed into mushrooms [108]. There are a number of agro-residues including wheat straw, rice straw, cotton stalks, sugarcane bagasse, cassava bagasse, banana pseudostems, wheat bran, sawdust, wood chips, peanut shells, coffee pulp and husk, sunflower seed, cottonseed hulls, and corn cobs which possess some potential chemical properties that make them suitable substrates for SSF [107, 109]. According to Uthandi et al. [110], ample amounts of agro-residues are generated upon processing of agricultural crops including wheat, paddy, maize, and sugarcane. About 0.75 tons of straw is generated per one ton of paddy, 300 kg of bagasse is generated per one ton of sugarcane, and an equal proportion of stover is generated upon corn processing.

However, the types and composition of the substrate utilized for mushroom production directly or indirectly impose its impact on the growth of mycelia, mushroom yield as well as nutritional and medicinal properties of produced mushrooms. Mushrooms are cultivated around the world for many years. Commercial worldwide mushroom production data are incorporated in Fig. 19.3.

Different edible mushrooms have been consumed for long for their nutritional values, flavor, and aroma. These mushrooms are nothing but the fruiting bodies of the white-rot or sometimes brown-rot fungi.

Mushrooms are a good source of carbohydrates, lipids, proteins along with fibers and minerals; thus they provide substantial health benefits to the consumers. The nutritional properties of edible mushrooms are summarized in Table 19.4.

Such nutritionally rich mushrooms are produced upon solid-state fermentation of various residue-based substrates by white-rot/brown-rot mushroom fungi. For the

Table 19.4 Nutritional values of edible mushrooms

Mushroom	Nutritional value				References
	Protein (g/100 g)	Carbohydrate (g/100 g)	Lipid (g/100 g)	Fibers (g/100 g)	
Pleurotus	17–42	37–48	0.5–5	24–31	[112]
Ganoderma	13.3	82.3	3.0	–	[113]
Agaricus	56.3	37.5	2.7	–	[88]
Tricholoma	18.1–30.5	31.1–52.3	2–6.6	30.1	[114]
Phellinus	6.11–10.9	75.04–83.82	0.96–15.86	–	[115]
Grifola	21.1	58.8	3.1	10.1	[116]
Auricularia	7.2	88.6	1.7	–	[117]
Lentinus	26.3	65.1	2.3	–	[118]
Cordyceps	21.9	24.2	8.2	–	[119]
Flammulina	3.9–17.8	8.6–70.8	1.8–2.9	–	[118]

bio-based production (SSF) of such mushrooms, availability of the lignocellulosic biomass substrate is the primary element. In the field, large amounts of residues are generated, but in contrast very small amounts are utilized for different purposes. The total availability of agro-residues for biological processing is dependent on various parameters, including total production, moisture content, leftover residues on soil to maintain soil organic content, animal feed in terms of grazing, and other agro-activities [120].

As fungal growth is influenced by nutrient availability, the production of mushrooms is influenced by the chemical composition of the lignocellulosic substrate used. Moreover, some other macro- and micro-elements including potassium, calcium, phosphorus, magnesium, manganese, sulfur, iron, zinc, boron, and molybdenum occur in agro-residues which help the growth of fungi at lower concentrations.

Fungi efficiently utilize these complex agro-industrial residues with the help of an array of enzyme system. These enzyme systems possess various enzymes that catabolize the degradation of cellulose (cellulase), hemicellulose (hemicellulase), lignin (laccase, lignin peroxidase, manganese peroxidase) as well as other accessory enzymes such as xylanase, hydrolase, endo- and exoglucanase, cellobiohydrolase, and glucosidase. These enzymes convert complex substrates into utilizable nutrients which favors the growth of fungi. In addition to the nutrients, there are certain factors such as moisture, water activity, temperature, and light that regulate the growth of fungal mycelia as well as fruiting [121].

Thus, agro-industrial wastes are extensively used for the cultivation of mushrooms around the world.

19.4.3 Agro-industrial Residues in Animal Feedstock Preparation

Animal nutrition is one of the rising concerns worldwide. Agricultural, dairy, and food industry waste products including lignocellulosic waste, vegetables, fruits, whey as well as waste from sugar processing industries have the great potential solution toward animal nutrition issues.

Agricultural residues such as bagasse, straw, husk, cobs, and stovers are argued to be the most abundant agricultural waste [122]. Although being abundantly generated, their availability as animal feed is limited. Animals may get good amounts of cellulose and hemicellulose from these agricultural wastes, but their application as an animal feed is constrained due to lack of proteins, oils, vitamins, and minerals. Thus in order to use these residues as an animal feed, they must be enriched to increase the nutrients, especially protein content. However, nutrient enrichment can be done with the help of microbes like bacteria and fungi.

Solid-state fermentation (SSF) is one of the most preferred biotechnological processes that can be employed for the nutritional enrichment of agricultural wastes to be used as animal feed. SSF provides convenient environment and conditions which are similar to that of the natural conditions and thus stimulates the growth of filamentous fungi. Various fungi that have already been used in preparing enriched animal feeds are listed in Table 19.5.

Many researchers have also worked on protein enrichment of various fruit and vegetable wastes. Kot et al. [140] reprocessed the potato pulp and wastewater for the enrichment of protein content. Villas-Boas et al. [141] have reported about 500% enrichment of protein using *Pleurotus ostreatus* in apple pomace and furthermore employed in food preparation. Moreover, to increase the nutritional value and digestibility, various food grade enzymes have also been employed as supplements or additives during animal feed preparation [142].

Although agro-industrial raw materials are available and feed production is cheaper, the logistic costs constrain faster development of such production technologies, further making the product costly. Moreover, this technology is somewhat labor intensive and time consuming. There is thus a challenge in improving and developing economically feasible, less time-consuming, on-site applicable and scalable technologies for enrichment and production of animal feed.

19.4.4 Agro-industrial Residues in Production of Bioactive Secondary Metabolites

Secondary metabolites are synthesized by microorganisms during the late log or stationary phase of their life cycle, which are not essential for reproduction and normal growth of an organism. These secondary metabolites may be antibiotics, polysaccharides, alkaloids, terpenes, and steroids, which are economically significant industrial products [143].

The most commonly used technology for production of secondary metabolites is microbial fermentation. Among different fermentation methods, submerged

Table 19.5 Preparation of protein-enriched agro-industrial residues using fungi

Fungal species	Agro-industrial substrate	Improvement in feed product	References
Microsphaeropsis spp., Coprinus fimetarius, Phanerochaete chrysosporium, Pleurotus ostreatus, Neurospora sitophila, Trametes spp., Ganoderma spp., Trichoderma spp.	Wheat bran/straw/corn stover/buckwheat/millet, sugar beet pulp, citrus waste, mustard straw, bean straw, agave bagasse Perennial grass	Protein-enriched straw/feed Edible and medicinal mushrooms	[123–129]
Pleurotus spp.	Coffee pulp; coffee husk	Protein-enriched biomass	[130, 131]
Rhizopus oligosporus, Penicillium funiculosum, Myrothecium verrucaria	Apple pomace, apple waste, apple pulp, grape	Protein-rich fungi and feed	[132–134]
Rhizopus oryzae, Cephalosporium eichhorniae, *Lentinus* spp.	Cassava wastes (peels; slurry; bagasse; wastewater), cassava Tubers, & starch	Animal feed and food, protein-enriched biomass, edible mushroom; protein-enriched flour	[135–137]
Aspergillus niger	Lupin meal	Improvement of fish growth, feed performance, and gut morphology	[138]
Pleurotus eryngii	Wheat bran	Increase in lignocellulolytic enzymes activity	[29]
Beauveria bassiana, Fusarium flocciferum	Olive cake	Increase in protein content and decrease in phenolic and flavonoids content	[139]

fermentation (SmF) is widely used because the scale-up procedures are quite easier and production parameters are easy to manipulate. Moreover, the control over various parameters such as pH, temperature, and nutritional requirements is better under SmF. However, to use agro-industrial residues for the production of secondary metabolites, SSF must be opted. SSF is comparatively a labor-intensive process, but on the other hand it provides natural environment to the organisms for biological processes.

Fungi are quite ideal for SSF system than bacteria, as they have capability to grow and flourish under lower water activities. There are a large number of fungi capable of producing such biologically active secondary metabolites using agro-industrial residues (Table 19.6).

Table 19.6 Preparation of fungal secondary metabolites from agro-industrial residues

Agro-industrial substrate	Fungal species	Secondary metabolite	References
Barley	*Cephalosporium acremonium*	Cephalosporin	[144, 145]
Corn	*Fusarium moniliforme*	Zearalenone	[146]
Rice, rice bran, rice husk	*Metarhizium anisopliae*	Destruxins A and B	[147]
Sugarcane bagasse	*Claviceps purpurea, C. fusiformis*	Ergot alkaloids	[148, 149]
Sugarcane bagasse	*Penicillium chrysogenum*	Penicillin	[150]
Wheat bran	*Tolypocladium inflatum*	Cyclosporin A	[148]
Wheat bran	*P. brevicompactum*	Mycophenolic	[151]
Wheat bran, corn cob, cassava flour, grains	*Gibberella fujikuroi, Fusarium moniliforme*	Gibberellic acid	[152, 153]
Wheat, oat, rice, maize, peanuts	*Aspergillus oryzae, A. parasiticus*	Aflatoxin	[154, 155]
Wheat, oat, rice, maize, peanuts	*A. oryzae*	Ochratoxin	[156]

There are several factors that influence the production of bioactive secondary metabolites under submerged fermentation. These factors include type of agro-industrial residue, particle size, moisture content, temperature as well as aeration and agitation of substrate.

Although being greatly available, the agro-industrial wastes are underutilized for the commercial production of industrially significant secondary metabolites. However, the use of agricultural waste must be prioritized by the industries for large-scale production of such metabolites as it is evident that higher concentration of the products can be achieved through SSF. Moreover, the substrate is even cheaper than that of media components used in SmF.

19.4.5 Agro-industrial Residues in Production of Biofuels

Finite fuel resources, increasing demand for fuels around the globe, and emission of toxic greenhouse gases upon combustion lead researchers to find some alternative sources; and biofuels are one of those. Biofuels are also known as bio-based fuels; they are made from a combination of biomass and chemicals, considered as the most economical transportation fuel. There are two major types of biofuels: gaseous and liquid biofuels. Biofuels are fast processing fuels, unlike the fossil fuels which are created through slow geological processes. Agro-industrial waste biomass is used as raw material for the production of biofuels. It can be made from different sources, such as plants, industrial wastes, domestic and commercial crops. The carbon content of the fuel varies depending on the environment and the emission levels

[157]. The global annual biofuel production reached 161 billion liters in 2019, which is about 6% higher than that of 2018 [158]. In 2000 the global biofuel production was about 187 thousand barrels of oil equivalent per day, which rise to 1677 thousand barrels of oil equivalent per day in 2020. Salidini et al. [159] classified biofuels into four classes (first-, second-, third-, and fourth-generation biofuels), based on their feedstocks and production methods.

First-generation biofuels are made from edible biomass like starch (from potatoes, wheat, barley, and corn) or sugars (from sugarcane and sugar beet). They initially showed promise in reducing fossil fuel combustion and lowering atmospheric CO_2 levels as crops grow using edible crops as feedstocks, as well as the impacts on croplands, biodiversity, and food supply [160]. Biodiesel (bio-esters), bioethanol, and bio-gas are examples of first-generation biofuels.

Diesel fuel, with a chemical formula ranging from $C_{10}H_{20}$ to $C_{15}H_{28}$ with an average molecular weight of 168 (amu), is a popular liquid petroleum fuel for transportation [161]. Biodiesel is made by transesterifying oils or fats and can be used as a vehicle fuel in its pure form (B100), but it is most commonly employed as a diesel additive to reduce particulates, carbon monoxide, and hydrocarbon emissions from diesel-powered vehicles. It is generally made up of fatty acid methyl (or ethyl) esters chemically (FAMEs) [162]. Biodiesel is also safe to handle and transport because it is nontoxic and biodegradable, with a flash point of around 148 °C, compared to 52 °C for petroleum diesel fuel [163]. Bioethers (also known as fuel ethers) are additives to gasoline that increase the octane number. They can be used to replace petro-ethers and increase the performance of engines [164]. Bioethers can also significantly reduce engine wear and hazardous exhaust emissions. They are created when bioethanol reacts with iso-olefins such as isobutylene. Wheat and sugar beet are the most common sources of bioethers [165].

Second-generation biofuels are produced using more sustainable techniques. When second-generation biofuels are burned, the net carbon emitted or consumed is neutral or even negative. Agriculture waste, poplar trees, willow and eucalyptus, sugarcane bagasse, switchgrass, corn cobs, and wood are only a few examples of cheap and plentiful nonedible abandoned materials that can be utilized as biofuel feedstock [166].

Second-generation bioethanol is prepared by hydrolysis and subsequent fermentation of agro-industrial residues. It can also be made by thermochemical methods, such as gasification followed by fermentation or a catalyzed reaction [167]. However, these processes are complicated by the difficulty of biomass breakdown, the release of various types of sugars after the breakdown of hemicellulose and cellulose polymers, the need to ferment these sugars with suitable organisms, which may necessitate genetic engineering, and the cost of collecting and storing low-density lignocellulosic feedstocks [168]. In the lignocellulosic conversion process, there are four main operational steps: pretreatment, hydrolysis, fermentation, and product separation or distillation [169] (Fig. 19.4).

Energy crops, agricultural waste, and wood residual wastage are all examples of second-generation feedstock that can be used to make biodiesel. Jatropha, *Aleurites moluccana*, salmon oil, Rubber tree, *Madhuca longifolia*, tobacco seed, sea mango,

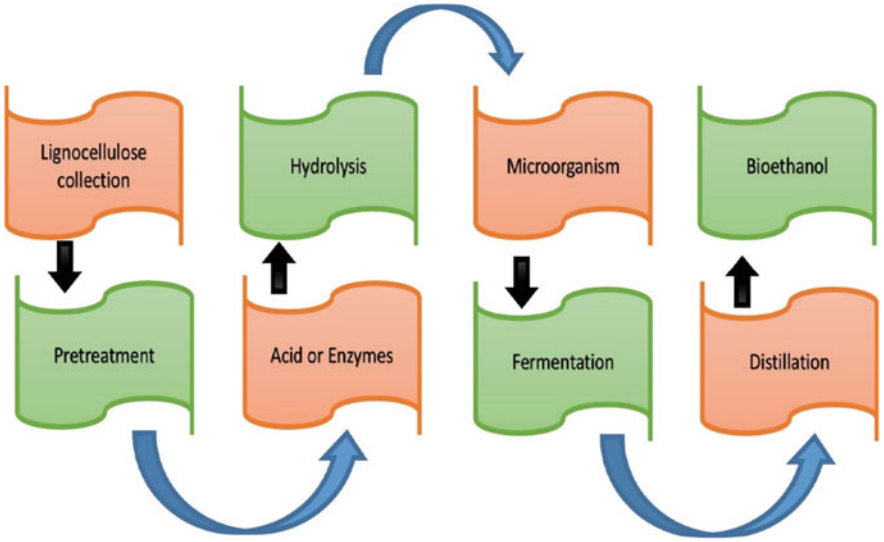

Fig. 19.4 Schematic representation of bioethanol production from LCB

and jojoba oil are the most popular energy crops used for this purpose. The waste from cooking oils, nonedible oil crops, restaurant grease, and animal fats can be included as feedstock for the production of second-generation biodiesel [170].

In the third generation of biofuels, a variety of microorganisms are used as feedstocks [115]. The most frequent form for biofuel production is promising microalgae because of their photosynthetic ability, producing specific chemicals and nutritious items. When compared to fossil-based oil produced by quick pyrolysis of wood, microalgae-based bio-oil has a high heating value, low density, and low viscosity [171]. Genetically engineered microorganisms such as microalgae, yeast, fungus, and cyanobacteria are used as sources in fourth-generation biofuels [172].

19.4.5.1 Biohydrogen from Agro-industrial Residues

Hydrogen is a very high energy (122 kJ/g) yielding fuel as compared to ethanol or methane. When combusted it liberates water in place of greenhouse gases. Using light as a primary source of energy, photoautotrophic growing bacteria and microalgae, the hydrogenase enzyme splits water into hydrogen and oxygen. Various solid agricultural wastes such as black strap molasses, rice straw as well as liquid waste from rice winery and sugar mills have been successfully employed for the production of hydrogen fuel [173]. The application of potato steam peels has also been studied for hydrogen production. The total hydrogen and acetate production using only glucose as a source of carbon and equivalent amount of sugars in potato steam peel hydrolysate (prepared by the action of amylase and glucoamylase) was assessed. The study revealed that higher hydrogen production and maximum hydrogen productivity (218 mM and 11.7 mmol/L.h, respectively) occurred from the peels than that from glucose (130 mM and 10.7 mmol/L.h, respectively) [174].

19.4.5.2 Bioethanol from Agro-industrial Residues

Bioethanol is produced by simple fermentation of cheaper and renewable agricultural carbohydrate feedstock using yeasts as biocatalysts. Various common sugar feedstocks such as sweet sorghum, sugar beet tubers, and sugarcane stalks have been successfully employed for the production of bioethanol. The yeast cell produces two enzymes, namely zymase and invertase, which mediate the fermentation process. Initially, the lignocellulosic biomass is subjected to chemical or enzymatic pretreatment for the conversion of cellulose and hemicellulose into complex sugars. Then invertase enzyme produced by the yeast cell converts these complex sugars into simple fermentable sugars which will be fermented further to produce crude ethanol and carbon dioxide. This crude ethanol contains significant amounts of water and thus it is subjected to fractional distillation (vaporization) to remove water and pure ethanol (95%) is further obtained upon distillation. Bioethanol production from renewable and cheap agro-industrial residues reduces greenhouse gas emissions like CO_X, SO_X, and NO_X as well as eliminates smog. Thippi, corn steep liquor, switchgrass (*Panicum virgatum*), potato waste, oat straw, rice straw, wheat straw as well as Ami-ami solution (Brewer's yeast autolysate and fish soluble waste) have been globally used as raw materials for bioethanol production [175, 176].

19.4.5.3 Biodiesel from Agro-industrial Residues

The use of oil from peanuts was initially demonstrated by Rudolf Diesel in his self-designed engine during the World Exhibition held in Paris in 1900. After that, numerous trials have been made to establish the potential of triglycerides as an alternative to diesel. Anyhow, high viscosity and poor low temperature properties of triglycerides were the limitations to be used directly in diesel engines. These limitations can be overcome by modifying the properties of vegetable oils that resemble that of petrodiesel. Biodiesel is mono alkyl esters produced upon transesterification of triglycerides with alcohol (methanol) in the presence of chemical catalysts like acid/alkali or biological catalysts like enzymes. Biodiesel is generally prepared from vegetable oils like palm oil, rice bran oil, rapeseed oil, sunflower oil, and soybean oil as well as from animal fats. The type of vegetable oil to be used in the biodiesel production mainly depends upon the abundance near the production site. Biodiesel from animal fats has several advantages over biodiesel from vegetable origin. Biodiesel produced from animal fats has a high cetane number due to less amounts of unsaturated fatty acids than as compared to vegetable oils; and a higher cetane number emits lower NOx gases [177]. Moreover, biodiesel from animal fats also has a high calorific value [178]. Researchers have reported that blends of soybean/beef tallow biodiesel presented higher oxidative stabilities as compared to biodiesel from soybean oil only [179].

Increasing prices of crude oils and cost-effectiveness of major biofuel technologies have globally accelerated extensive utilization of agro-industrial residues for the production of alternative biofuels. Gaseous biofuels (biomethane and biohydrogen) as well as liquid biofuels (bioethanol and biodiesel) have evolved as a potential alternative to the diminishing fuel resources. Biotechnologies used for the production of biofuels from agro-industrial wastes have potential in reducing

greenhouse gas emissions and release of toxic pollutants, thus saving the environment and partly solving the global fuel crisis. By considering the sustainability and transformative power of biotechnologies in biofuel production, the "second industrial revolution" can be enabled that the society now requires.

19.4.6 Application of Agro-industrial Residues in Pharmaceutical Industry

Pharmaceutical industries are one of the essential industries in the world and are the backbone of many developing nations including India. According to a study carried out by IQVIA, the pharmaceutical sector should experience market growth between 3 and 6% worldwide over the next five years, which amounts to exceeding 1.5 trillion dollars in total value by the year 2023 [180]. India currently ranks third in the world in terms of volume and 14th in terms of value for pharmaceutical compound production. The country has vast domestic pharmaceutical sector with a robust network of 3000 pharmaceuticals businesses and 10,500 production units as of 2022 [181]. With the constant rise in population along emergence and re-emergence of diseases, the demand of pharmaceutical products will increase in near future which puts pressure on supply of raw products to meet the demand. Agricultural residue can provide alternate raw material for many pharmaceutical products and help meeting the demand in future [182].

In recent times there has been an increase in manufacturing antimicrobial compounds to combat respiratory disease, infections, and many other physiological conditions in humans [183]. However the excessive usages of synthetic pharmaceutical products have led to unforeseeable consequences for humans such as resistance in microorganism and side effects of drugs [184]. These limitations have sparked interest among research community to investigate alternate routes for drug preparations and use of bioactive compounds from agricultural residues [185]. Edible fruits are heavily utilized in food industries and their waste is known to possess raw materials that can be used in drug manufacturing. Currently, citrus fruits (Lemon, Orange, limes, etc.) are receiving special attention from researchers for their antiviral, anti-inflammatory, antibacterial, and antifungal properties [186]. Agri-food wastes such as peels, pomace, and seeds can assist in enhancing bioavailability of various drugs as they are a rich source of nutrients as well as phytochemical compounds. They are an excellent source of organic acid, sugars, and polyphenolic compounds such as flavonoids and anthocyanins which have proven antibacterial, antifungal, anti-inflammatory, immunomodulatory, and antioxidant properties [187].

Constant emergence and re-emergence of viral diseases such as hepatitis, Ebola, MERS-CoV (Middle East respiratory syndrome), SARS-CoV (severe acute respiratory syndrome), H7N9 (avian influenza virus), and Crimean-Congo fever have put increasing pressure on the entire health sector and industry to focus research on finding alternative medicine with antiviral properties. In this regard, bioactive compounds present in agro-wastes such as tangeretin, nobiletin, and hesperidin have shown great potential of antiviral properties in terms of infected cell activity

reduction and viral multiplication inhibition [188]. In the light of recent disease outbreaks such as Zika virus and COVID-19, many alterative drugs and natural compounds like Arbiol, Remdesivir, and Lopinavir are now being investigated for their direct antiviral activities [189].

19.4.7 Miscellaneous Applications of Agro-industrial Residues

Agricultural residues such as seeds, fruit peels, fruit skins, and cereal husks have the capability to enhance sensory and nutritional characteristics when partially substituted with 10% to 30% of wheat and corn flour in preparation of bread. Furthermore fruit peels are a novel source of colorant which adds in color, flavor, antioxidant and anti-inflammatory properties to bakery products [182]. Natural phytophenolic compounds are valuable products because of their medicinal properties and some of the important sources are peels of lemons, oranges, and grapefruits. Notably peel residues from apples, peaches, pears, and nectarines contains twice the amount of total phytophenolic compounds compared to fruit pulp [190]. Similarly grape seeds and skins which are waste products from the grape juice and wine industry are also sources of several phytophenolic compounds such as mono, oligo, and polymeric proanthocyanidins [191]. Dietary fibers are an essential component of diet as they have a role in the prevention of diabetes, obesity, atherosclerosis, heart diseases, colon cancer, and colorectal cancer. Among the LCB components, hemicellulose and pectin possess significant metal binding capability, which has a positive role in metabolism. The utilization of by-products or wastes from industrial processing of fruit and vegetables, i.e., apple, currant, citrus fruit, carrot, tomato, melon, or spinach pomace, is convenient and cost-effective and enables rational management of troublesome wastes.

Agricultural wastes and residues are biodegradable in nature and although most common use of it is as the feedstock, huge amount of it goes straight to landfills and dumps. This waste can be used to obtain composite materials which can be used in manufacturing many products and as a building material [192–194]. The raw agricultural residues can be used to produce nano-composite materials for a wide range of applications. The common ingredients of nanocomposites (NCs) are nanocellulose, nanoscale carbon-based materials, and nanosilica. They are currently utilized in industrial sector, agriculture, pharmaceutical, and remediation of pollutants. The most potent agro-residues for preparation of nanocomposites are banana peels, orange peels, wheat whiskers, straw, cotton stalks, corn stalks, coconut shells, almond shells, corn silk, rice husks, oil palm empty fruit bunches, bagasse, peanut hulls, and ginger rhizome [192, 194, 195]. NCs can be fabricated using hydrothermal carbonization, sol-gel method, co-precipitation, polymer solution casting, phase inversion technique, ball milling, and direct compounding methods (Fig. 19.5) [196].

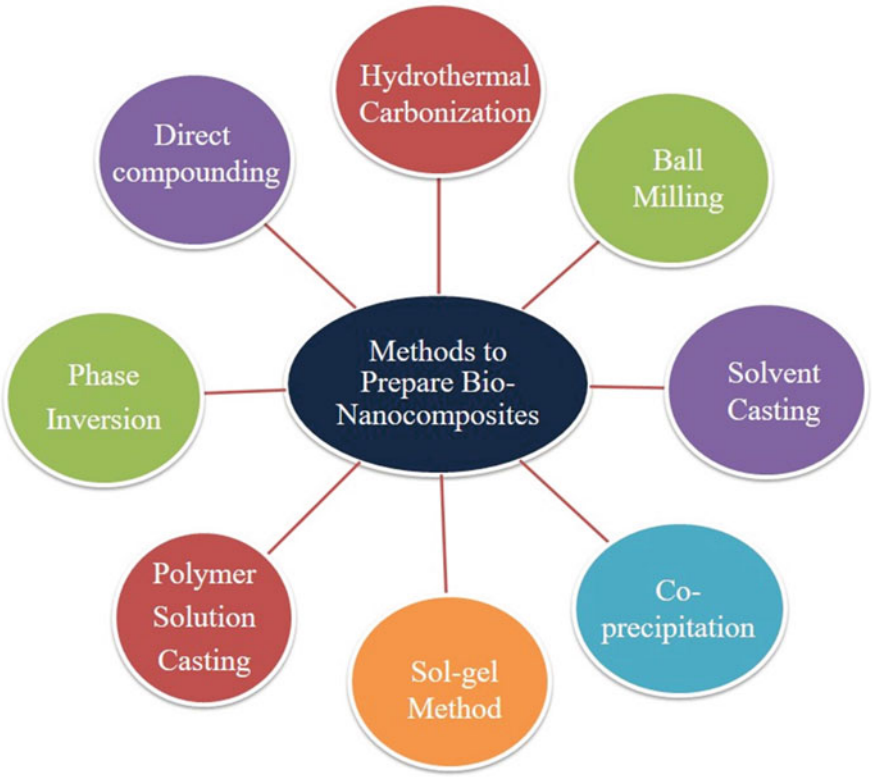

Fig. 19.5 Different methods for the preparation of bio-nanocomposites from LCB

19.5 Conclusion and Future Prospects

Agro-industrial residues are abundantly found in nature and have been widely distributed all over the world through trading, import-export, and direct industrial applications. However the major portion of these residues is not utilized efficiently, and it ends up in landfills and waste dumps. While common agro-residues like cereal straws and cobs are used in animal fodders, other residues like oil cakes, pseudostem, bagasse, and fruit shells and peels are not suitable as fodder; thus most of it ends up as a solid waste. In the present scenario regarding economy and environmental changes in the world, it is important that not only these agro-residues be recycled and treated but also be utilized for the production of renewable energy and value-added products. In recent times, fungi have been the most suitable organism for the production of many value-added products from agro-residue due to their robustness and ability to survive in adverse environmental conditions and different ecological niches. Furthermore, fungi can produce a wide array of enzymes which can break down complex LCB residues and generate value-added products.

This chapter summarizes all types of agro-residues and their potential industrial applications by using bio-based refinery approach. One of the important components of the biorefinery concept is the fractionation of complex lignocellulosic biomass residues which help in the separation of major components of LCB residue such as cellulose, hemicellulose, and lignin with minor phytochemicals. While cellulose and hemicellulose can be used for the production of animal feed, industrially significant enzyme, and organic fertilizers, lignin component is used for the production of laccase class of enzymes and phytophenolic compounds. The direct implementation of LCB residues as a substrate has also been used for the production of industrially significant enzymes, secondary metabolites, and mushroom production. Phytochemicals from LCB residues have vast potential to generate many bioactive compounds which have antimicrobial, nutraceutical, antioxidant, and many other pharmaceutically significant properties. Currently the energy and food demand has sharply increased due to population rise, exploitation of natural resources, and environmental changes. Thus it is the need of the hour to switch to renewable resources and switch to green technologies with low carbon emission and high recyclability.

Agro-industrial residues are the most prominent resource which are easily available in nature. The limitations of bioactive compounds and emerging drug resistance of pathogens have also put the industry in dire need of looking for alternative medicinal compounds. Agro-residues can be used to generate many novel bioactive compounds which can be used for the purpose of alternative medicine. Current trends in research suggest that in the coming years, utilization of agro-residues will play a major role in renewable energy and product industries.

References

1. Zohuri B, McDaniel P (2021) Introduction to energy essentials: insight into nuclear, renewable, and non-renewable energies. Academic Press, London
2. Andersson Ö, Börjesson P (2021) The greenhouse gas emissions of an electrified vehicle combined with renewable fuels: life cycle assessment and policy implications. Appl Energy 289:116621
3. Motto M, Sahay S (2022) Energy plants (crops): potential natural and future designer plants. In: Handbook of biofuels. Academic Press, London, pp 73–114
4. Mankar AR, Pandey A et al (2021) Pretreatment of lignocellulosic biomass: a review on recent advances. Bioresour Technol 334:125235
5. Yousuf A, Pirozzi D, Sannino F (2020) Fundamentals of lignocellulosic biomass. In: Lignocellulosic biomass to liquid biofuels. Academic Press, London, pp 1–15
6. Yousuf A (2012) Biodiesel from lignocellulosic biomass–prospects and challenges. Waste Manag 32(11):2061–2067
7. Qaisar K, Nawaz A et al (2021) Advances in valorization of lignocellulosic biomass towards energy generation. Catalysts 11(3):309
8. Liguori R, Amore A, Faraco V (2013) Waste valorization by biotechnological conversion into added value products. Appl Microbiol Biotechnol 97(14):6129–6147
9. Pandey A, Soccol CR et al (2000) Biotechnological potential of agro-industrial residues. I: sugarcane bagasse. Bioresour Technol 74(1):69–80

10. Silva TAL, Zamora HDZ et al (2018) Effect of steam explosion pretreatment catalysed by organic acid and alkali on chemical and structural properties and enzymatic hydrolysis of sugarcane bagasse. Waste Biomass Valorization 9(11):2191–2201
11. Contato AG, de Oliveira TB et al (2021) Prospection of fungal lignocellulolytic enzymes produced from jatoba (*Hymenaea courbaril)* and tamarind (*Tamarindus indica*) seeds: scaling for bioreactor and saccharification profile of sugarcane bagasse. Microorganisms 9(3):533
12. Torgbo S, Quan VM, Sukyai P (2021) Cellulosic value-added products from sugarcane bagasse. Cellulose 28(9):5219–5240
13. Khoo SC, Ma NL et al (2022) Valorisation of biomass and diaper waste into a sustainable production of the medical mushroom Lingzhi *Ganoderma lucidum*. Chemosphere 286:131477
14. Abe MM, Branciforti MC et al (2022) Production and assessment of the biodegradation and ecotoxicity of xylan-and starch-based bioplastics. Chemosphere 287:132290
15. Kumar A, Kumar V, Singh B (2021) Cellulosic and hemicellulosic fractions of sugarcane bagasse: potential, challenges and future perspective. Int J Biol Macromol 169:564–582
16. Pandey A, Soccol CR et al (2000) Biotechnological potential of agro-industrial residues. II: cassava bagasse. Bioresour Technol 74(1):81–87
17. da Cruz Kerber CM, Rasbold LM et al (2021) Production of hemicellulolytic enzymes by a novel *Trichoderma koningiopsis* 2OI2A1M and its application in the saccharification of barley bagasse. Waste Biomass Valorization 12(11):5949–5958
18. Travalini AP, Lamsal B et al (2019) Cassava starch films reinforced with lignocellulose nanofibers from cassava bagasse. Int J Biol Macromol 139:1151–1161
19. Vasudhaudupa A, Gowda B, Shivanna MB (2021) Influence of non-edible oil-cakes and their composts on growth, yield and *Alternaria* leaf spot disease in chilli. Int J Recycl Org Waste Agri 11:301
20. Sivaramakrishnan S, Gangadharan D (2009) Edible oil cakes. In: Biotechnology for agro-industrial residues utilisation. Springer, Dordrecht, pp 253–271
21. Ancuța P, Sonia A (2020) Oil press-cakes and meals valorization through circular economy approaches: a review. Appl Sci 10(21):7432
22. Balakrishnan M, Jeevarathinam G et al (2021) Optimization and scale-up of α-amylase production by *aspergillus oryzae* using solid-state fermentation of edible oil cakes. BMC Biotechnol 21(1):1–11
23. Szymczak T, Cybulska J et al (2021) Various perspectives on microbial lipase production using agri-food waste and renewable products. Agriculture 11(6):540
24. Tai WY, Tan JS et al (2019) Comprehensive studies on optimization of cellulase and xylanase production by a local indigenous fungus strain via solid state fermentation using oil palm frond as substrate. Biotechnol Prog 35(3):e2781
25. Singh S, Kaur A, Gupta A (2021) Tannase production through solid-state fermentation of *Shorea robusta* deoiled seed cake: an industrial biomass using Aspergillus flavus TF-8 for potential application in gallic acid synthesis. Biomass Conv Bioref:1–11. https://doi.org/10.1007/s13399-021-01634-3
26. Elumalai P, Lim JM et al (2020) Agricultural waste materials enhance protease production by *Bacillus subtilis* B22 in submerged fermentation under blue light-emitting diodes. Bioprocess Biosyst Eng 43(5):821–830
27. Jatuwong K, Kumla J et al (2020) Bioprocessing of agricultural residues as substrates and optimal conditions for phytase production of chestnut mushroom, Pholiota adiposa, in solid state fermentation. J Fungi 6(4):384
28. Logarušić M, Gaurina Srček V et al (2021) Protein hydrolysates from flaxseed oil cake as a media supplement in CHO cell culture. Resources 10(6):59
29. Wang Q, Zheng H et al (2017) Optimization of inexpensive agricultural by-products as raw materials for bacitracin production in *bacillus licheniformis* DW2. Appl Biochem Biotechnol 183(4):1146–1157

30. Saranraij P, Sivasakthivelan P et al (2022) Microbial fermentation technology for biosurfactants production. In: Microbial surfactants: volume 2: applications in food and agriculture. CRC Press, Boca Raton, pp 25–43
31. Chen H, Yang Y, Zhang J (2009) Biotechnological potential of cereal (wheat and rice) straw and bran residues. In: Biotechnology for agro-industrial residues utilisation. Springer, Dordrecht, pp 327–340
32. Fontaine D, Eriksen J, Sørensen P (2020) Cover crop and cereal straw management influence the residual nitrogen effect. Eur J Agron 118:126100
33. Singhal A, Konttinen J, Joronen T (2021) Effect of different washing parameters on the fuel properties and elemental composition of wheat straw in water-washing pre-treatment. Part 1: effect of washing duration and biomass size. Fuel 292:120206
34. Ambatkar N, Jadhav DD et al (2021) Functional screening and adaptation of fungal cultures to industrial media for improved delignification of rice straw. Biomass Bioenergy 155:106271
35. Sekifuji R, Le VC et al (2021) Solubility and physical composition of rice husk ash silica as a function of calcination temperature and duration. Int J Recycl Org Waste Agri 10(1):19–27
36. Maulinda L, Husin H et al (2021) Fast pyrolysis corn husk for bio-oil production. IOP Conf Ser Mater Sci Eng 1098(2):022007
37. Zou Y, Fu J et al (2021) The effect of microstructure on mechanical properties of corn cob. Micron 146:103070
38. Ding R, Yang X et al (2021) Development of mycelium materials incubating *Pleurotus Ostreatus* fungi with different substrates composed of poplar sawdust and cottonseed hull (Preprint)
39. Pellegrini VDOA, Ratti RP et al (2022) Differences in chemical composition and physical properties caused by industrial storage on sugarcane bagasse result in its efficient enzymatic hydrolysis. Sustain Energy Fuels 6(2):329–348
40. Alio MA, Marcati A et al (2021) Modeling and simulation of a sawdust mixture-based integrated biorefinery plant producing bioethanol. Bioresour Technol 325:124650
41. Rudakiya DM, Gupte A (2019) Assessment of white rot fungus mediated hardwood degradation by FTIR spectroscopy and multivariate analysis. J Microbiol Methods 157:123–130
42. Morales-Martínez JL, Aguilar-Uscanga MG et al (2021) Optimization of chemical pretreatments using response surface methodology for second-generation ethanol production from coffee husk waste. Bioenergy Res 14(3):815–827
43. Dinesha P, Kumar S, Rosen MA (2019) Biomass briquettes as an alternative fuel: a comprehensive review. Energ Technol 7(5):1801011
44. Kamm B, Gruber PR, Kamm M (2006) Biorefineries-industrial processes and products, vol 2. Wiley-VCH, Weinheim
45. Kumar P, Singh A, Kumar D (2020) An overview of working models and approaches to climate smart livestock farming. Int J Life Sci Appl Sci 2(1):28–36
46. Yan X, Gan L, Chen M (2022) Development of forage industry in China. In: Research progress on forage production, processing and utilization in China. Springer, Singapore, pp 29–41
47. Zang Q, Chen X et al (2022) Improving crude protein and methionine production, selective lignin degradation and digestibility of wheat straw by Inonotus obliquus using response surface methodology. J Sci Food Agric 102:1146
48. Liu L (2006) The fractionation of straw and its high value conversion [doctoral dissertation]. Institute of Process Engineering, Chinese Academy of Sciences, Beijing
49. Ho SC, Kuo CT (2014) Hesperidin, nobiletin, and tangeretin are collectively responsible for the anti-neuroinflammatory capacity of tangerine peel (Citri reticulatae pericarpium). Food Chem Toxicol 71:176–182
50. Piatti-Farnell L (2016) Banana: a global history. Reaktion Books, London
51. Tak MK, Kumar V et al (2015) Correlation of banana cv Grand Naine with growth and yield aspect. J Plant Develop Sci 7(1):1–6

52. Venkateshwaran N, Elayaperumal A (2010) Banana fiber reinforced polymer composites-a review. J Reinf Plast Compos 29(15):2387–2396
53. Jirukkakul N (2019) Physical properties of banana stem and leaf papers laminated with banana film. Walailak J Sci Technol 16(10):753–763
54. Pappu A, Patil V et al (2015) Advances in industrial prospective of cellulosic macromolecules enriched banana biofibre resources: a review. Int J Biol Macromol 79:449–458
55. Thorat RL, Bobade HP (2018) Utilization of banana pseudo-stem in food applications. Int J Agric Eng 11(SP):86–89
56. Li K, Fu S et al (2010) Analysis of the chemical composition and morphological structure of banana pseudo-stem. Bioresources 5(2):576–585
57. Diarsa M, Gupte A (2020) Optimization and extraction of natural dye from *Tagetes Erecta* and dyeing of cotton and silk fabric using Banana (*Musa* sp.) Pseudo Stem Sap. J Nat Fibers 19: 4443–4455
58. Bhatnagar R, Gupta G, Yadav S (2015) A review on composition and properties of banana fibers. Int J Sci Eng Res 6(5):49–52
59. Vellaichamy M, Gaonkar PV (2017) Biological treatment of banana pseudostem fibre: effect on softening and mechanical properties. Int J Curr Microbiol Appl Sci 6(5):1268–1274
60. Diarsa M, Gupte A (2021) Preparation, characterization and its potential applications in Isoniazid drug delivery of porous microcrystalline cellulose from banana pseudostem fibers 3Biotech. 11(7):1–13
61. Ventura-Cruz S, Flores-Alamo N, Tecante A (2020) Preparation of microcrystalline cellulose from residual Rose stems (Rosa spp.) by successive delignification with alkaline hydrogen peroxide. Int J Biol Macromol 155:324–329
62. Geissdoerfer M, Pieroni MP et al (2020) Circular business models: a review. J Clean Prod 277: 123741
63. Meyer V, Basenko EY et al (2020) Growing a circular economy with fungal biotechnology: a white paper. Fungal Biol Biotechnol 7(1):1–23
64. Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83(1):1–11
65. Sun Y, Cheng JJ (2005) Dilute acid pretreatment of rye straw and bermudagrass for ethanol production. Bioresour Technol 96(14):1599–1606
66. Maguire NA, Kuhmann T et al (2022) Statistical mixture designs for media development with agro-industrial residues–supporting the circular bioeconomy. EFB Bioecon J 2:100023
67. Ravindran R, Hassan SS et al (2018) A review on bioconversion of agro-industrial wastes to industrially important enzymes. Bioengineering 5(4):93
68. Chhaya U, Gupte A (2013) Possible role of laccase from *Fusarium incarnatum* UC-14 in bioremediation of Bisphenol A using reverse micelles system. J Hazard Mater 254:149–156
69. Patel H, Gupte S et al (2014) Purification and characterization of an extracellular laccase from solid-state culture of *Pleurotus ostreatus* HP-1. 3Biotech 4(1):77–84
70. Pinheiro VE, Michelin M et al (2020) *Trametes versicolor* laccase production using agricultural wastes: a comparative study in Erlenmeyer flasks, bioreactor and tray. Bioprocess Biosyst Eng 43(3):507–514
71. Yang H, Wu X et al (2019) Fungal transformation of graphene by white rot fungus *Phanerochaete chrysosporium*. Chemosphere 216:9–18
72. Shaheen R, Asgher M et al (2017) Immobilized lignin peroxidase from *Ganoderma lucidum* IBL-05 with improved dye decolorization and cytotoxicity reduction properties. Int J Biol Macromol 103:57–64
73. Vandana T, Kumar SA et al (2019) Purification, characterization, and biodelignification potential of lignin peroxidase from immobilized *Phanerochaete chrysosporium*. Bioresources 14(3):5380–5399
74. Colonia BSO, Woiciechowski AL et al (2019) Pulp improvement of oil palm empty fruit bunches associated to solid-state biopulping and biobleaching with xylanase and lignin peroxidase cocktail produced by Aspergillus sp. LPB-5. Biores Technol 285:121361

75. Pandya JJ, Gupte A (2012) Production of xylanase under solid-state fermentation by *Aspergillus tubingensis* JP-1 and its application. Bioprocess Biosyst Eng 35(5):769–779
76. Hu Y, Du C et al (2018) Optimisation of fungal cellulase production from textile waste using experimental design. Process Saf Environ Protect 118:133–142
77. Lodha A, Pawar S, Rathod V (2020) Optimised cellulase production from fungal co-culture of *Trichoderma reesei* NCIM 1186 and *Penicillium citrinum* NCIM 768 under solid state fermentation. J Environ Chem Eng 8(5):103958
78. Dange VU, Harke S (2018) Production and purification of pectinase by fungal strain in solid-state fermentation using agro-industrial bioproduct. Int J Life Sci Res 6(4):85–93
79. Thamvithayakorn P, Phosri C et al (2019) Utilization of oil palm decanter cake for valuable laccase and manganese peroxidase enzyme production from a novel white-rot fungus, *Pseudolagarobasidium* sp. PP17-33. 3Biotech 9(11):1–10
80. Olaniyi OO, Arotupin DJ et al (2014) Kinetic properties of purified [beta]-Mannanase from *Penicillium italicum*. Br Microbiol Res J 4(10):1092
81. Bharathiraja S, Suriya J et al (2017) Production of enzymes from agricultural wastes and their potential industrial applications. In: Advances in food and nutrition research, vol 80. Academic Press, Amsterdam, pp 125–148
82. Rebhun M, Wasser SP, Hadar Y (2005) Use of agro-industrial waste for production of laccase and manganese peroxidase from white-rot basidiomycetes. Int J Med Mushrooms 7(3): 459–460
83. do Rosario Freixo M, Karmali A et al (2008) Production of laccase and xylanase from *Coriolus versicolor* grown on tomato pomace and their chromatographic behaviour on immobilized metal chelates. Process Biochem 43(11):1265–1274
84. Birhanli E, Yesilada O (2013) The utilization of lignocellulosic wastes for laccase production under semisolid-state and submerged fermentation conditions. Turkish J Biol 37:450–456
85. Wang F, Hu JH et al (2014) Enhanced laccase production by *Trametes versicolor* using corn steep liquor as both nitrogen source and inducer. Bioresour Technol 166:602–605
86. An Q, Liu ZY et al (2021) Laccase activity from *Pleurotus ostreatus* and *Flammulina velutipes* strains grown on agro-and forestry residues by solid-state fermentation. Bioresources 16(4): 7337
87. de Oliveira Júnior SD, dos Santos Gouvêa PR et al (2022) Production of lignocellulolytic enzymes and phenolic compounds by *Lentinus strigosus* from the Amazon using solid-state fermentation (SSF) of guarana (*Paullinia cupana*) residue. Appl Biochem Biotechnol 194: 2882–2900
88. Wahlström R, Rahikainen J, Kruus K, Suurnäkki A (2014) Cellulose hydrolysis and binding with *Trichoderma reesei* Cel5A and Cel7A and their core domains in ionic liquid solutions. Biotechnol Bioeng 111:726–733
89. Picart P, Diaz P, Pastor FIJ (2007) Cellulases from two *Penicillium* sp. strains isolated from subtropical forest soil: production and characterization. Lett Appl Microbiol 45:08–113
90. Waghmare PR, Kshirsagar SD et al (2014) Production and characterization of cellulolytic enzymes by isolated Klebsiella sp. PRW-1 using agricultural waste biomass. Emir J Food Agric 26(1):44–59
91. Olajuyigbe FM, Ogunyewo OA (2016) Enhanced production and physicochemical properties of thermostable crude cellulase from *Sporothrix carnis* grown on corn cob. Biocatal Agric Biotechnol 7:110–117
92. Perez CL, Casciatori FP, Thoméo JC (2019) Strategies for scaling-up packed-bed bioreactors for solid-state fermentation: the case of cellulolytic enzymes production by a thermophilic fungus. Chem Eng J 361:1142–1151
93. Rayhane H, Josiane M et al (2019) From flasks to single used bioreactor: scale-up of solid state fermentation process for metabolites and conidia production by *Trichoderma asperellum*. J Environ Manag 252:109496
94. Laothanachareon T, Bunterngsook B, Champreda V (2022) Profiling multi-enzyme activities of *Aspergillus niger* strains growing on various agro-industrial residues. 3Biotech 12(1):1–16

95. Bastawde KB (1992) Xylan structure, microbial xylanases, and their mode of action. World J Microbiol Biotechnol 8(4):353–368
96. Gawande PV, Kamat MY (1999) Production of *Aspergillus* xylanase by lignocellulosic waste fermentation and its application. J Appl Microbiol 87(4):511–519
97. Milagres AMF, Santos E et al (2004) Production of xylanase by *Thermoascus aurantiacus* from sugar cane bagasse in an aerated growth fermentor. Process Biochem 39(11):1387–1391
98. Patel K, Prajapati K (2014) Xylanase production by *Cladosporium* sp. from agricultural waste. Int J Curr Res Acad Rev 2(12):84–90
99. Cunha L, Martarello R et al (2018) Optimization of xylanase production from Aspergillus foetidus in soybean residue. Enzyme Res 2018:6597017
100. Ismail SA, Nour SA, Hassan AA (2022) Valorization of corn cobs for xylanase production by *Aspergillus flavus* AW1 and its application in the production of antioxidant oligosaccharides and removal of food stain. Biocatal Agric Biotechnol 41:102311
101. Rangarajan V, Rajasekharan M et al (2010) Pectinase production from orange peel extract and dried orange peel solid as substrates using *Aspergillus niger*. Int J Biotechnol Biochem 6(3): 445–453
102. Satapathy S, Soren JP et al (2021) Industrially relevant pectinase production from *Aspergillus parvisclerotigenus* KX928754 using apple pomace as the promising substrate. J Taibah Uni Sci 15(1):347–356
103. Gaur VK, Sharma P et al (2022) Production of biosurfactants from agro-industrial waste and waste cooking oil in a circular bioeconomy: an overview. Bioresour Technol 343:126059
104. Lindsay MA, Granucci N et al (2022) Identification of new natural sources of flavour and aroma metabolites from solid-state fermentation of agro-industrial by-products. Metabolites 12(2):157
105. Manzini PFL, Islas-Samperio JM et al (2022) Sustainability assessment of solid biofuels from agro-industrial residues case of sugarcane bagasse in a Mexican sugar mill. Sustainability 14(3):1711
106. Raina D, Kumar V, Saran S (2022) A critical review on exploitation of agro-industrial biomass as substrates for the therapeutic microbial enzymes production and implemented protein purification techniques. Chemosphere 294:133712
107. Šelo G, Planinić M et al (2021) A comprehensive review on valorization of agro-food industrial residues by solid-state fermentation. Foods 10(5):927
108. Grimm D, Wösten HA (2018) Mushroom cultivation in the circular economy. Appl Microbiol Biotechnol 102(18):7795–7803
109. Manan MA, Webb C (2019) Insights into physical characterization of solid state fermentation: from preliminary knowledge to practical application. J Biotech Res 10:271–282
110. Uthandi S, Kaliyaperumal A et al (2021) Microbial biodiesel production from lignocellulosic biomass: new insights and future challenges. Crit Rev Environ Sci Technol 52:2197–2225
111. https://www.fao.org/faostat/en
112. Iuchi T, Hosaka T et al (2015) Influence of treatment with extracts of *Hypsyzigus marmoreus* mushroom on body composition during obesity development in kk-ay mice. J Nutr Sci Vitaminol 61(1):96–100
113. Wachtel-Galor S, Yuen J et al (2011) *Ganoderma lucidum* (Lingzhi or Reishi). In: Herbal medicine: biomolecular and clinical aspects, 2nd edn. CRC Press, Boca Raton
114. Ding Q, Yang D et al (2016) Antioxidant and anti-aging activities of the polysaccharide TLH-3 from *Tricholoma lobayense*. Int J Biol Macromol 85:133–140
115. Lee JK, Song JH, Lee JS (2010) Optimal extraction conditions of antiobesity lipase inhibitor from *Phellinus linteus* and nutritional characteristics of the extracts. Mycobiology 38(1):58–61
116. Cheung PCK (2010) The nutritional and health benefits of mushrooms. Nutr Bullet 35(4): 292–299
117. Nhi NNY, Hung PV (2012) Nutritional composition and antioxidant capacity of several edible mushrooms grown in the Southern Vietnam. Int Food Res J 19(2):611–615

118. Gunawardena D, Bennett L et al (2014) Anti-inflammatory effects of five commercially available mushroom species determined in lipopolysaccharide and interferon-c activated murine macrophages. Food Chem 148:92–96
119. Heo JC, Nam SH et al (2010) Anti-asthmatic activities in mycelial extract and culture filtrate of *Cordyceps sphecocephala* J201. Int J Mol Med 26:351–356
120. Kosre A, Koreti D et al (2021) Current perspective of sustainable utilization of agro waste and biotransformation of energy in mushroom. In: Energy: crises, challenges and solutions. Wiley, Hoboken, pp 274–302
121. Orban A, Weber A et al (2021) Transcriptome of different fruiting stages in the cultivated mushroom *Cyclocybe aegerita* suggests a complex regulation of fruiting and reveals enzymes putatively involved in fungal oxylipin biosynthesis. BMC Genom 22(1):1–23
122. Ginni G, Kavitha S et al (2021) Valorization of agricultural residues: different biorefinery routes. J Environ Chem Eng 9(4):105435
123. Ivarsson E, Grudén M et al (2021) Use of faba bean (Vicia faba L.) hulls as substrate for *Pleurotus ostreatus*–potential for combined mushroom and feed production. J Clean Prod 313: 127969
124. Katileviciute A, Plakys G et al (2019) A sight to wheat bran: high value-added products. Biomolecules 9(12):887
125. Nepomuceno R, Brown CMB, Brown MB (2021) A paradigm shift towards sustainability: utilizing agricultural waste as a valuable resource in various agricultural endeavours. In: Agri-based bioeconomy: reintegrating trans-disciplinary research and sustainable development goals. CRC Press, Boca Raton, pp 131–142
126. Nur-Nazratul FMY, Rakib MRM et al (2021) Enhancing in vitro ruminal digestibility of oil palm empty fruit bunch by biological pre-treatment with *Ganoderma lucidum* fungal culture. PLoS One 16(9):e0258065
127. Tišma M, Žnidaršič-Plazl P et al (2021) *Trametes versicolor* in lignocellulose-based bioeconomy: state of the art, challenges and opportunities. Bioresour Technol 330:124997
128. Vuong MD, Thanh NT et al (2021) Protein enrichment of cassava-based dried distiller's grain by solid state fermentation using *Trichoderma Harzianum* and *Yarrowia Lipolytica* for feed ingredients. Waste Biomass Valorization 12(7):3875–3888
129. Yanti G, Jamarun N, Astuti T (2021) Quality improvement of sugarcane top as animal feed with biodelignification by *Phanerochaete chrysosporium* fungi on in-vitro digestibility of NDF, ADF, cellulose and hemicellulose. J Phys Conf Ser 1940(1):012063
130. Carmen MT, Lorena ZC et al (2020) Coffee pulp: an industrial by-product with uses in agriculture, nutrition and biotechnology. Rev Agri Sci 8:323–342
131. Ritota M, Manzi P (2019) Pleurotus spp. cultivation on different agri-food by-products: example of biotechnological application. Sustainability 11(18):5049
132. Aganbi E, Anigboro AA, Tonukari NJ (2020) Changes in glucose, amylase and soluble proteins levels in solid state fermented yam (Dioscorea Spp.) peels by Rhizopus oligosporus. Nigerian J Sci Environ 18(1)
133. Dantroliya S, Joshi C et al (2022) Creating wealth from waste: an approach for converting organic waste in to value-added products using microbial consortia. Environ Technol Innov 25:102092
134. Ganesh KS, Sridhar A, Vishali S (2022) Utilization of fruit and vegetable waste to produce value-added products: conventional utilization and emerging opportunities—a review. Chemosphere 287:132221
135. Barnharst T, Sun X et al (2021) Enhanced protein and amino acids of corn–ethanol co-product by *Mucor indicus* and *Rhizopus oryzae*. Bioprocess Biosyst Eng 44(9):1989–2000
136. Mao L, Cone JW et al (2020) Wheat bran addition improves *Ceriporiopsis subvermispora* and *Lentinula edodes* growth on wheat straw, but not delignification. Animal Feed Sci Technol 259:114361
137. Spalvins K, Blumberga D (2019) Single cell oil production from waste biomass: review of applicable agricultural by-products. Agron Res 17(3):833–849

138. Bowyer PH, El-Haroun ER et al (2020) Benefits of a commercial solid-state fermentation (SSF) product on growth performance, feed efficiency and gut morphology of juvenile Nile tilapia (*Oreochromis niloticus*) fed different UK lupin meal cultivars. Aquaculture 523:735192
139. Chebaibi S, Grandchamp ML et al (2019) Improvement of protein content and decrease of anti-nutritional factors in olive cake by solid-state fermentation: a way to valorize this industrial by-product in animal feed. J Biosci Bioeng 128(3):384–390
140. Kot AM, Pobiega K et al (2020) Biotechnological methods of management and utilization of potato industry waste - a review. Potato Res 63(3):431–447
141. Villas-Bôas SG, Esposito E, de Mendonca MM (2003) Bioconversion of apple pomace into a nutritionally enriched substrate by *Candida utilis* and *Pleurotus ostreatus*. World J Microbiol Biotechnol 19(5):461–467
142. Mamma D, Kourtoglou E, Christakopoulos P (2007) Fungal multienzyme production on industrial by-products of the citrus-processing industry. Bioresour Technol 99:2373–2383
143. Prajapati D, Bhatt A et al (2021) Mushroom secondary metabolites: chemistry and therapeutic applications. Int J Pharm Sci Res 12(11):5677–5689
144. Singh Nigam P (2009) Production of bioactive secondary metabolites. In: Biotechnology for agro-industrial residues utilisation: utilisation of agro-residues. Springer, Dordrecht, pp 129–145
145. Yadav AN, Kour D et al (2019) Metabolic engineering to synthetic biology of secondary metabolites production. In: New and future developments in microbial biotechnology and bioengineering. Elsevier, Amsterdam, pp 279–320
146. Tyska D, Mallmann AO et al (2021) Multivariate method for prediction of fumonisins B1 and B2 and zearalenone in Brazilian maize using near infrared spectroscopy (NIR). PLoS One 16(1):e0244957
147. Meera Y, Nivedita R et al (2020) Bioconversion of lignocellulose materials using different pre-treatment strategies. Res J Chem Environ 24:12
148. Aljammas HA (2021) Utilization of agroindustrial residues for the production of microbial secondary metabolites: general aspects. In: Green sustainable process for chemical and environmental engineering and science. Elsevier, pp 57–74
149. Doohan F, Zhou B (2018) Fungal pathogens of plants. In: Fungi biology and applications, vol 3. Wiley, Hoboken, pp 355–386
150. Singh S, Khajuria R (2018) *Penicillium* enzymes for the textile industry. In: New and future developments in microbial biotechnology and bioengineering. Elsevier, Amsterdam, pp 201–215
151. El-Sayed ESR, Ahmed AS, Ismaiel AA (2019) Agro-industrial byproducts for production of the immunosuppressant mycophenolic acid by *Penicillium roqueforti* under solid-state fermentation: enhanced production by ultraviolet and gamma irradiation. Biocatal Agric Biotechnol 18:101015
152. da Silva LRI, de Andrade CJ et al (2021) Solid-state fermentation in Brewer's spent grains by *Fusarium fujikuroi* for gibberellic acid production. Biointerface Res Appl Chem 11:13042–13052
153. Werle LB, Abaide ER et al (2020) Gibberellic acid production from *Gibberella fujikuroi* using agro-industrial residues. Biocatal Agric Biotechnol 25:101608
154. Daba GM, Mostafa FA, Elkhateeb WA (2021) The ancient koji mold (*Aspergillus oryzae*) as a modern biotechnological tool. Biores Bioprocess 8(1):1–17
155. Mahmoodzadeh HH, Hamzeh PS et al (2020) The effect of Propolis on inhibition of *Aspergillus parasiticus* growth, aflatoxin production and expression of aflatoxin biosynthesis pathway genes. J Environ Health Sci Eng 18(1):297–302
156. Perrone G, Susca A et al (2012) Biodiversity of *Aspergillus* species in some important agricultural products. Stud Mycol 59:53
157. Alalwan HA, Alminshid AH, Aljaafari HA (2019) Promising evolution of biofuel generations. Subject review. Renew Energy Focus 28:127–139
158. https://www.iea.org/reports/transport-biofuels

159. Saladini F, Patrizi N et al (2016) Guidelines for emergy evaluation of first, second and third generation biofuels. Renew Sust Energ Rev 66:221–227
160. Rodionova MV, Poudyal RS et al (2017) Biofuel production: challenges and opportunities. Int J Hydrog Energy 42(12):8450–8461
161. Mahmudul HM, Hagos FY et al (2017) Production, characterization and performance of biodiesel as an alternative fuel in diesel engines–a review. Renew Sust Energ Rev 72:497–509
162. Yusoff MFM, Xu X, Guo Z (2014) Comparison of fatty acid methyl and ethyl esters as biodiesel base stock: a review on processing and production requirements. J Am Oil Chem Soc 91(4):525–531
163. Demirbas A (2009) Production of biodiesel fuels from linseed oil using methanol and ethanol in non-catalytic SCF conditions. Biomass Bioenergy 33(1):113–118
164. Negm NA, Sayed GH et al (2017) Biodiesel production from one-step heterogeneous catalyzed process of Castor oil and Jatropha oil using novel sulphonated phenyl silane montmorillonite catalyst. J Mol Liquids 234:157–163
165. Bagheri S, Julkapli NM, Zolkepeli RAA (2017) Catalytic conversion on lignocellulose to biodiesel product. In: Nanotechnology for bioenergy and biofuel production. Springer, Cham, pp 207–229
166. Trabelsi ABH, Zaafouri K et al (2018) Second generation biofuels production from waste cooking oil via pyrolysis process. Renew Energy 126:888–896
167. Wyman CE (2018) Ethanol production from lignocellulosic biomass: overview. In: Handbook on bioethanol. Routledge, London, pp 1–18
168. Vyas P, Kumar A, Singh S (2018) Biomass breakdown: a review on pretreatment, instrumentations and methods. Front Biosci 10(815):10–2741
169. Jönsson LJ, Martín C (2016) Pretreatment of lignocellulose: formation of inhibitory by-products and strategies for minimizing their effects. Bioresour Technol 199:103–112
170. Bhuiya MMK, Rasul MG et al (2016) Prospects of 2nd generation biodiesel as a sustainable fuel—part: 1 selection of feed stocks, oil extraction techniques and conversion technologies. Renew Sust Energ Rev 55:1109–1128
171. Silva CM, Ferreira AF et al (2016) A comparison between microalgae virtual biorefinery arrangements for bio-oil production based on lab-scale results. J Clean Prod 130:58–67
172. Vassilev SV, Vassileva CG (2016) Composition, properties and challenges of algae biomass for biofuel application: an overview. Fuel 181:1–33
173. Nath K, Kumar A, Das D (2005) Hydrogen production by Rhodobacter sphaeroides strain OU 001 using spent media of *Enterobacter cloacae* strain DM11. Appl Microbiol Biotechnol 68(4):533–541
174. Claassen PAM, De Vrije T et al (2004) Biological hydrogen production from sweet sorghum by thermophilic bacteria. In 2nd World Conference on Biomass for Energy, Industrial and Climate Protection, Rome, Italy
175. Asli MS (2010) A study on some efficient parameters in batch fermentation of ethanol using *Saccharomyces cerevesiae* SC1 extracted from fermented siahe sardasht pomace. Afr J Biotechnol 9(20):2906
176. Patle S, Lal B (2008) Investigation of the potential of agro-industrial material as low cost substrate for ethanol production by using *Candida tropicalis* and *Zymomonas mobilis*. Biomass Bioenergy 32(7):596–602
177. Banković-Ilić IB, Stojković IJ et al (2014) Waste animal fats as feedstocks for biodiesel production. Renew Sustain Energy Reviews 32:238–254
178. Adewale P, Dumont MJ, Ngadi M (2015) Recent trends of biodiesel production from animal fat wastes and associated production techniques. Renew Sust Energ Rev 45:574–588
179. Pereira GG, Garcia RK et al (2017) Soybean and soybean/beef-tallow biodiesel: a comparative study on oxidative degradation during long-term storage. J Am Oil Chem Soc 94(4):587–593
180. IQVIA. The Global Use of Medicine in 2019 and Outlook to 2023 (2019) https://www.iqvia.com/insights/theiqvia-institute/reports/the-global-use-of-medicine-in-2019-and-outlook-to-2023

181. https://www.ibef.org/industry/pharmaceutical-india.aspx
182. Osorio LLDR, Flórez-López E, Grande-Tovar CD (2021) The potential of selected agri-food loss and waste to contribute to a circular economy: applications in the food, cosmetic and pharmaceutical industries. Molecules 26(2):515
183. Fair RJ, Tor Y (2014) Antibiotics and bacterial resistance in the 21st century. Perspect Med Chem 6:PMC-S14459
184. Aminov R (2017) History of antimicrobial drug discovery: major classes and health impact. Biochem Pharmacol 133:4–19
185. Peanparkdee M, Iwamoto S (2019) Bioactive compounds from by-products of rice cultivation and rice processing: extraction and application in the food and pharmaceutical industries. Trends Food Sci Technol 86:109–117
186. Xu DP, Zheng J et al (2016) Extraction of natural antioxidants from the *Thelephora ganbajun* mushroom by an ultrasound-assisted extraction technique and evaluation of antiproliferative activity of the extract against human cancer cells. Int J Mol Sci 17(10):1664
187. Roy S, Lingampeta P (2014) Solid wastes of fruits peels as source of low cost broad spectrum natural antimicrobial compounds-furanone, furfural and benezenetriol. Int J Res Engi Technol 3(7):273–279
188. Tang K, He S et al (2018) Tangeretin, an extract from citrus peels, blocks cellular entry of arenaviruses that cause viral hemorrhagic fever. Antivir Res 160:87–93
189. Bellavite P, Donzelli A (2020) Hesperidin and SARS-CoV-2: new light on the healthy function of citrus fruits. Antioxidants 9(8):742
190. Gorinstein S, Martín-Belloso O et al (2001) Comparison of some biochemical characteristics of different citrus fruits. Food Chem 74(3):309–315
191. Shrikhande AJ (2000) Wine by-products with health benefits. Food Res Int 33(6):469–474
192. Jawaid M, Paridah MT, Saba N (2017) Lignocellulosic fibre and biomass-based composite materials: processing, properties and applications. Woodhead Publishing, Kidlington
193. Rajinipriya M, Nagalakshmaiah M et al (2018) Importance of agricultural and industrial waste in the field of nanocellulose and recent industrial developments of wood based nanocellulose: a review. ACS Sustain Chem Eng 6(3):2807–2828
194. Sangeetha J, Thangadurai D et al (2017) Production of bionanomaterials from agricultural wastes. In: Nanotechnology. Springer, Singapore, pp 33–58
195. Jampílek J, Kráľová K (2020) Preparation of nanocomposites from agricultural waste and their versatile applications. In: Multifunctional hybrid nanomaterials for sustainable agri-food and ecosystems. Elsevier, Amsterdam, pp 51–98
196. Ucankus G, Ercan M et al (2018) Methods for preparation of nanocomposites in environmental remediation. In: New polymer nanocomposites for environmental remediation. Elsevier, Amsterdam, pp 1–28

Design Strategies for Mycelium-Based Composites

20

Adrien Rigobello and Phil Ayres

Abstract

Mycelium-based composites (MBC) can be designed utilising a wide range of lignocellulosic substrates and widely distributed versatile ligninolytic fungi. While a wide range of mechanical behaviour has already been reported in the past 15 years, showing potential to obtain viable products for a variety of uses; no systematic description of the engineering parameters has been established till date. We review carbohydrate-active enzyme (CAZyme) activities of fungal species, lignocellulosic substrate chemical profile at cellular level, wetting characteristics, substrate aggregate and composition characteristics. We identify three principal strategies for designing MBC: supplementation, densification and composition, and discuss them regarding outstanding reports from the state-of-the-art. We report on solid-state fermentation supplements having significant effect on fungal CAZymes activities (e.g. monosaccharide, nitrogen, ash, pH of buffer). State-of-the-art designs and process control promote specific enzymatic activities independent of species genomics; systematically investigating supplementation, densification and composition design strategies in the future may lead to both a widening and deepening of the available material qualities, along with a focus on developments around functional poles. Additionally, future reproducibility studies of MBC development reports may both improve the overall market readiness and public adoption of MBC solutions and valorise the wealth of material and design semiotic properties that the versatility and affordability of MBC systems support.

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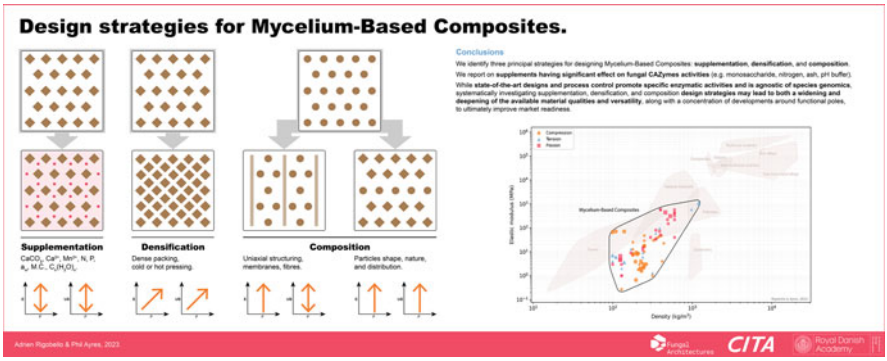
Graphical Abstract



Keywords

Solid-state fermentation · Ligninolytic fungi · Mycelium-based composites · CAZymes · Biocomposites · Biomaterials

Highlights

- We identify three principal strategies for designing mycelium-based composites: supplementation, densification and composition.
- We report on supplements and wetting metrics having significant effect on fungal CAZyme activities and mycelia phenotypic expression.
- We report on the composition strategies having significant effect on mechanical response of mycelium-based composites.
- We present state-of-the-art mechanical reports comparatively from species and substrate specifics.
- Systematic investigations in future may lead to widening and deepening available material qualities along with a focus on functional poles.

20.1 Introduction

Inherited from the nineteenth- to early twentieth-century method of fungal strain transfer by lignocellulosic solid-state fermentation (SSF) [1], mycelium-based composite (MBC) cultivation protocols can be based on a wide range of substrates containing lignocellulosic polymers using versatile ligninolytic fungi. Widely available basidiomycota have been instrumental in developing composites with a wide range of mechanical performance, showing potential to obtain viable products for a variety of uses. However, no systematic description of the engineering parameters has been established till date. Although the first MBC commercial venture was established in 2006 (Ecovative Design LLC), an aggressive patent strategy

combined with an inertia in explaining the engineering-driving principles of MBC has resulted in a very low market readiness and market permeability of the material solutions. With a global urgent need to adopt low-energy and low-impact alternatives to existing detrimental material systems on a scale, we argue that rationalising and systematising approaches to analysing MBC systems is necessary to actualise their potential and facilitate market readiness.

As composites, MBC are made of a matrix phase that corresponds to a continuous and foam-like or elastomer-like mycelium, and a dispersed phase composed of particles and/or fibres. Additionally, surface or volumetric components can be used to compose the substrate of a composite and modify its properties. Fungal decay is functionalised as a binding method between elements of the dispersed phase. From this generic definition, we can appreciate the sheer breadth of the material design space, and can identify the main material characteristics for defining the cultivation protocol and specifying composite properties:

- Enzyme arrays of fungal species,
- Substrate chemical profile,
- Substrate wetting characteristics,
- Substrate aggregate characteristics,
- Substrate density and composition.

The resulting composite properties depend on the SSF model that is adopted. While tacit knowledge of MBC craft is sufficient for reaching a satisfactory output, thanks to the competitive colonisation dynamics of the fungi in use, the explanation and modelling of the driving parameters of MBC systems can foster a deeper engagement through engineering practice, thus extending the reach and effectiveness of material research and improving market readiness. Paste-like substrate design for fungal colonisation is not studied at present. The inner structure of a typical MBC is presented in Fig. 20.1.

We start by presenting a review of the MBC model from a ligninolytic fungi enzymatic activities perspective, and proceed to describe the biochemistry of

Fig. 20.1 Typical inner structure of an MBC

lignocellulosic substrates while correlating it to fungal enzymatic abilities. Building upon studies of fungal niches in the wild and their parasitic or saprotrophic behaviour in trees, we come to understand the hydrolytic abilities of wood in relation to their chemical composition and evolutionary stage as a primary parameter for predicting fungal colonisation. A comprehensive review of supplementation methods for influencing enzymatic activities is presented for ultimately adapting protocols to locally available resources. Furthermore, in describing the behaviour of MBC from this perspective, we review studies that have shown a clear correlation between the mycelial mechanical properties and their cultivation substrate chemical composition. While these properties have been demonstrated to be modifiable to result in foam-like or elastomer-like behaviour, nature of substrate aggregates (size, distribution and shape) are the principal contributors of composite strength and stiffness. Outstanding mechanical results from the MBC state-of-the-art are then discussed with regard to these insights, in an effort to describe the behaviour of composites. We identify three main substrate-based exploration strategies for the MBC system: densification (by dense packing, cold- or hot-pressing), composition (by introducing structuring elements) and supplementation (targeting mycelium properties, and based on chemical tuning of the substrate). Furthermore, we have analysed available data in published peer-reviewed studies to offer a critical review of the state-of-the-art, and conclude on the most significant engineering parameters and recommend research directions.

20.2 Enzymatic Activities of Ligninolytic Fungi

The species of *Trametes*, *Ganoderma* and *Pleurotus* are among the most frequently cited fungal families in MBC design [2]. *Schizophyllum commune* is a less investigated species but there is a continued interest [3], especially because it is one of the few mushroom-forming fungi for which genes have been inactivated by homologous recombination [4]. This strategy has proved useful for genetic studies and functional genomics [5]. Gene inactivation articulates the principles of homologous recombination for replacing an existing autologous gene with a designed heterologous gene. Furthermore, the recombinant DNA constructs of *S. commune* has been reported to express in other mushroom-forming fungi, such as *Pycnoporus cinnabarinus*, which supports its importance for fungal biology [4, 6]. *Irpex lacteus* has been used previously in MBC development [7], as in *Agaricus bisporus* [8] and *Fomes fomentarius* [9]. Other less investigated species have also been reported [2].

Wood-rotting fungi can be classified by their decay activity, which cover three main modes: white-rot (specified as selective delignification and simultaneous rot), brown-rot and soft-rot. Across the surveyed species, all of them are white-rot, ligninolytic fungi. They have the enzymatic ability to decompose all three principal polymer classes found in wood: lignin, hemicellulose and cellulose, but most often degrade primarily lignin and hemicellulose as reported in *Ganoderma lucidum* [10]. In such cases, these species qualify as selective delignifies, as opposed to simultaneous decomposers where all three polymer classes endure similar decay

rates. Brown-rot fungi use a two-step oxidative–enzymatic mechanism for the breakdown of cellulose and hemicellulose [11]. Their lignin degradation is limited to modification in lignin, although brown-rot fungi is a polyphyletic group evolved from at least seven white-rot lineages [12]; they reportedly lack more than 60% of the genes known to be present in white-rot species. Brown-rot species degrade cellulose and hemicellulose at a higher rate than white-rot species in laboratory studies [13]. This decay mode is most often encountered in coniferous trees. Soft-rot fungi commonly share the ability to demethylate. This mode is associated with Ascomycota and monokaryotic mycelia, and is distinctive for its tunnelling decay pattern through lignin layers to reach cellulose and hemicellulose-rich areas [14]. Lignicolous fungi can benefit from a variety of enzymatic strategies to ultimately fully decompose lignocellulosic substrates. The environmental conditions for enzyme activation define the species niche and their subsequent distribution. The principal wood-rot enzyme families are presented in Table 20.1.

Lignin linkages result from radical reactions, in contrast to polysaccharide polymerisation resulting from hydrogen bonding. Lignin, therefore, does not involve hydrolytic enzymes and cannot be used as a source of carbon or energy for most wood-rot fungi. Its decomposition serves primarily for the fungus to gain access to hemicellulose, pectin and cellulose compounds. Lignin depolymerisation is mostly initiated by aromatic ring oxidation by peroxidases (by use of H_2O_2 or R–OOH) [35], while other aromatic compounds in the cell structure are mostly catabolised by monooxygenases and dioxygenases. Peroxidases are heme proteins, such as lignin peroxidase (LiP) and manganese peroxidase (MnP). LiPs are known to oxidise phenolic aromatic substrates along with various nonphenolic lignin compounds, and other organic compounds with a high redox potential. MnP leads to H_2O_2 oxidising Mn^{2+} to Mn^{3+}, an oxidising compound to monomeric phenols. Mn^{3+} chelates permeate through wood cells and decay lignin selectively. These chelates cannot oxidise nonphenolic compounds, but radicals formed by Mn^{3+} oxidation can, in turn, oxidise benzyl alcohols and other diarylpropane structures. MnP can also peroxidise lipids [36]. Versatile peroxidase (VP), in addition to MnP and LiP, are enzymes that are Mn^{2+} specific, but that can oxidise phenolic and nonphenolic compounds in its absence (as with LiP). They are involved in oxidation of high redox potential aromatic substrates, thus reducing Mn^{2+}-independent oxidation but not affecting Mn^{2+}-dependent one [15]. Mn^{2+}-independent oxidation capabilities for phenols, small dye compounds and amines has also been described for MnP [35], and it is predicted from sequencing that similar divergent MnP may be present in *Trametes* spp., *Pleurotus* spp. and *A. bisporus* among other species [35, 37]. Hydrogen peroxide, necessary for LiP and MnP activities, is the product of fungal enzymatic activity. A peroxidase family with wide substrate specificity, active at pH of approximately 3, has been identified in fungi: dye-oxidising peroxidase (DyP). In addition to oxidising peroxidase substrates, it can perform on anthraquinones. Laccases are a very versatile family of enzymes found in nearly all fungi including those without lignin degrading abilities [38]. These enzymes perform functions ranging from synthesis of melanin and other pigments to conidia and fruiting body formation, and lignin decomposition [39]. These belong to a multicopper enzyme

Table 20.1 Extracellular enzymes in lignicolous fungi and their optimal activation temperature and medium pH [15–34]

Target polymer	Enzyme family	Reaction	Substrate	Temperature (° C)	pH
Lignin	Lignin peroxidase	Oxidation	Phenol, nonphenolic aromatics	28–40	3.0–4.5
	Manganese peroxidase	Oxidation	Manganese ions, phenol, small dye compounds, amines	23–40	5.0–7.0
	Versatile peroxidase	Oxidation	Manganese ions, phenol, nonphenolic aromatics	35	7.0
	Dye-oxidising peroxidase	Oxidation	High redox-potential dyes, nonphenolic lignin model compounds	28–70	3.0–7.0
	Laccase	Oxidation	Phenol, diphenol, aminophenol, aromatic compounds	35–50	4.0–5.0
Hemicellulose	Xyloglucanase	Hydrolysis	β-1,3-Glucans, carboxymethyl cellulose, xyloglucan oligosaccharides	37–75	3.0–9.4
	Xylanase	Hydrolysis	Glycosic bonds in xylan backbone	28–50	3.0–9.0
	Mannan-degrading	Hydrolysis	Mannan, galactomannan	45–92	2.4–7.5
	Polygalacturonidase	Hydrolysis	Pectin	–	–
	Pectin lyase	β-Elimination	Heavily methyl-esterified	50	8.0
	Pectate lyase	β-Elimination	Lower esterification and Ca^{2+}	30–90	5.0–11.5
	Cellobiose dehydrogenase	Oxidation	Cellobiose, cellodextrins, lactose	60	3.0–10.0
Cellulose	Cellobiohydrolase I	Hydrolysis	Crystalline cellulose	40–80	–
	Cellobiohydrolase II	Hydrolysis	Amorphous cellulose, β-xylan	50–63	4.0–6.0
	GH5 endoglucanase	Hydrolysis	Cellulose	45–65	4.5–7.0
	GH7 endoglucanase	Hydrolysis	Crystalline cellulose	45–65	4.5–7.0
	GH12 endoglucanase	Hydrolysis	Amorphous cellulose	45–65	4.5–7.0
	GH45 endoglucanase	Hydrolysis	Crystalline cellulose	45–65	7.0
	GH1 β-glucosidase	Hydrolysis	β-Linked oligosaccharides, aglycone-linked β-glucosides	25–50	5.0–7.0
	GH3 β-glucosidase	Hydrolysis	β-Glucan	25–50	5.0–7.0

family and catalyse the removal of an electron from phenolic hydroxylic groups to form phenoxy radicals, which then polymerise via radical coupling. Ultimately, the reactions lead to the presence of oxidised quinones and couples oligomeric products. Laccases also have wide substrate specificity and can act upon an extended range of phenols, diphenols, aminophenols and aromatic compounds. When their catalytic reaction is accompanied by demethylation, it may lead to ring cleavage. Tying lignin decomposition to cellulose and hemicellulose decay, cellobiose dehydrogenase (CDH) has the ability to oxidise cellobiose, cellodextrins and lactose [16]. Hemicellulose-degrading enzymes are capable of hydrolysing various polymers, β-1,4-galactosidase degrades xyloglucan, xylan, galactomannan and pectin [17]. More specific xyloglucanases, xylanases, mannan-degrading enzymes and pectinases can be found in all fungi to a varied extent. Typically, pectin can be hydrolysed by polygalacturonidases, but also cleaved by β-elimination by pectin and pectate lyases. Cellulose hydrolysis by fungal enzymes employs glycohydrolases (GH) by glycosic bond cleavage. Among GH families, cellobiohydrolase I (CBH I) is the major cellulase being produced in white- and soft-rots; it binds to crystalline cellulose and hydrolyses it into cellobiose. Cellobiohydrolase II (CBH II), found in all but brown-rot fungi, binds to amorphous cellulose and generates cellobiose from the non-reducing end of cellulose using acid catalysis of the β-1,4-glycosic bond. Endoglucanases are a major enzyme group capable of hydrolysing cellulose polymer from within the molecule rather than its end. The variety encountered in fungi refers to four GH families: GH5, GH7, GH12 and GH45. GH5 endoglucanase is a less studied family in fungi. GH7 endoglucanase displays a similar mechanism to CBH I, with the difference that it can hydrolyse from the middle of cellulose molecules. GH12 endoglucanases lack a carbohydrate-binding module (CBM). They are therefore unable to degrade crystalline cellulose but can hydrolyse it in its amorphous form. GH45 endoglucanase benefits from a CBM. β-Glucosidases, produced by all fungi, belong to GH1 and GH3 families. GH1 β-glucosidases are able to cleave soluble β-linked oligosaccharides from up to nine glucose residue chains and aglycone-linked β-glucosides, and can be competitively inhibited by the products of CDH activity. GH3 β-glucosidases can remove single glucosyl residues from the non-reducing ends of oligo- and polysaccharides such as β-D-glucans, β-1,3-D-glucans, β-1,4-D-glucans [40]. Lytic polysaccharide monooxygenases (LPMO) operating by oxidative cleavage have been shown to enhance cellulose degradation [41, 42].

Carbohydrate-active enzymes (CAZymes) have been classified into six principal families: auxiliary activities (AA), glycoside hydrolase (GH), glycosyltransferase (GT), polysaccharide lyase (PL), carbohydrate esterase (CE) and carbohydrate-binding module (CBM). We have summarised the genome distribution of *Trametes versicolor*, *Pleurotus ostreatus* PC15 and *G. lucidum* G0119 according to this nomenclature in Table 20.2 to exemplify specific species decaying modes. *G. lucidum* benefits from the widest known CAZymes array with 565 genes having been assigned to these functions [10, 44]. This is a well-known versatile fungus capable of selective delignification, as we have previously confirmed experimentally [45]. The actual decay strategies in selective delignification-capable fungi has been

Table 20.2 Genome distribution of CAZymes from selected species: *T. versicolor* (TV) [43], *P. ostreatus* PC15 (PO) [43] and *G. lucidum* G0119 (GL) [44]

CAZyme family	Enzymes	TV	PO	GL
AA	Laccase, LiP, MnP, VP, DyP, CDH, LPMO	89	114	96
CBM	GH5-GH7-GH45 endoglucanase, CBH I	47	82	33
GH	GH12 endoglucanase, β-glucosidase, xyloglucanase, xylanase, polygalacturonidase, mannan-degrading, CBH II	222	235	262
GT	Glycosyltransferases	85	67	72
PL	Pectin lyase, pectate lyase	9	23	11
CE	Carbohydrate esterases	19	28	91

correlated to wood species, lignin contents, temperature, pH and moisture content [46–50].

20.3 Lignocellulosic Substrates

Lignin is the most effective barrier to fungal decay as it prevents migration of low molecular weight diffusible agents (i.e. H_2O_2, veratryl alcohol, oxalate, Fe^{2+}, Fe^{3+}, Mn^{2+}, Mn^{3+}) for decomposing cellulose and hemicellulose. The nature of lignins is of considerable influence on the effectiveness of enzymatic activity. Lignins are polymers of phenylpropene units and they comprise guaiacyl, syringyl and *p*-hydroxyphenyl units. Gymnospermous woods (conifers) are almost exclusively composed of guaiacyl monomers, while angiospermous trees (hardwood) contain approximately equal shares of guaiacyl and syringyl monomers [51]. Similarly, different distributions of hemicelluloses are found in gymnospermous trees (galactoglucomannan, glucomannan and arabinoglucuronxylan are the principal ones) and in angiospermous trees (arabinogalactan, xyloglucan and various glucans are the principal compounds) [14]. Pectins are primarily made of β-1,4-D-galacturonase acid units and their methylesters, interrupted locally by 1,2-linked L-rhamnose units [52]. The primary constituent of wood is cellulose, which is made of repeating glucose units joined by β-1,4-glycosyl linkages. Cellulose represents about 50% of the wood cell wall, lignin 25%, hemicellulose 20–25% and pectin 1–4%. Hemp is often used in MBC practice. Their lignocellulosic components ratio is C:HC:L, which is close to 15:2:1, while their shives display a ratio closer to 2:1:1. The cultivation period and weather has been reported to cause differences in non-cellulosic composition [53].

The variety of wood structures and their chemical composition pairs with various decay strategies driven from the set of fungal CAZyme expression. These wood specifics can be understood from the evolutionary history of cell specialisation. Principal wood structures are presented in Fig. 20.2, reprinted [54]. Wood cells can be made of various specialised cells: tracheids, xylem ray parenchyma, axial parenchyma and vessels. Tracheids are found down to a first evolutionary stage of trees in gymnospermous woods, where they combine the tasks of water transport and

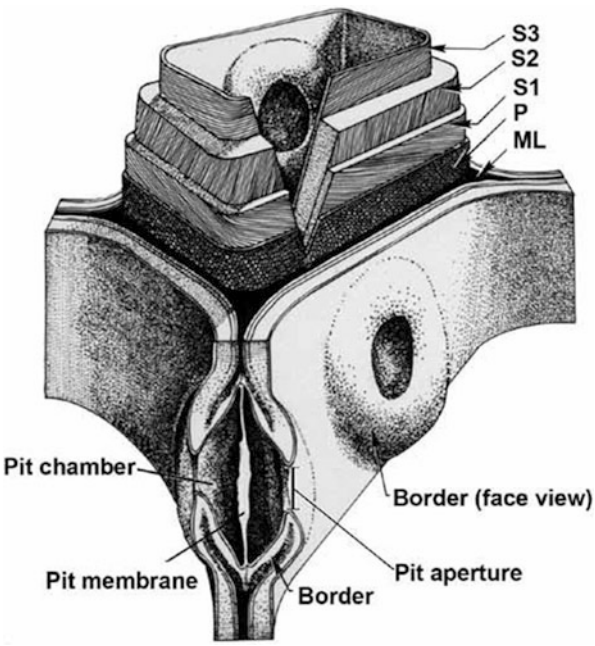


Fig. 20.2 Principal structures of soft- and hardwood; reprinted [54]

structural stiffness. In contrast, gymnospermous woods typically lack vessels and rarely have axial parenchyma. Their tracheid-to-xylem ray parenchyma ratio is most often in the vicinity of 9:1. They contain principally guaiacyl lignin, a product of coniferyl alcohol [55]. In more recent angiospermous woods, water is transported by vessels while tracheids perform structurally. Another evolutionary stage of water transport system in trees can be exemplified by European beech (*Fagus sylvatica*) and birch (*Betula pendula*). Their xylem includes vessels along with tracheids, the former being the only distinct location of guaiacyl lignin. Tracheids and parenchyma contain a fraction of syringyl lignin spread across the cell wall layers, making this secondary evolutionary wood structure attractive for white-rot fungi [14]. A third evolutionary stage can be illustrated by European oak (*Quercus robur*), for which the xylem is ring-porous, containing early and late wood vessels in bands within a thin-walled tracheid matrix. The strength of the wood comes from libriform fibres containing higher syringyl lignin, while tracheids have a higher guaiacyl content [56]. A late evolutionary stage is exemplified by sycamore (*Acer pseudoplanatus*) and ash (*Fraxinus* spp.), where syringyl dominates [57]. Here, vessels are dedicated to water transport, and are jacketed in living libriform fibres that are included amongst dead ones. Guaiacyl lignin has been reported to have a higher resistance to fungal decay [14], which has been corroborated by phylogenetic and experimental studies, showing that catalytic tryptophan presence in more recently evolved peroxidases (located in LiP and VP families) is more efficient at oxidising angiospermous woods, while others perform better in gymnospermous woods [58]. Early-

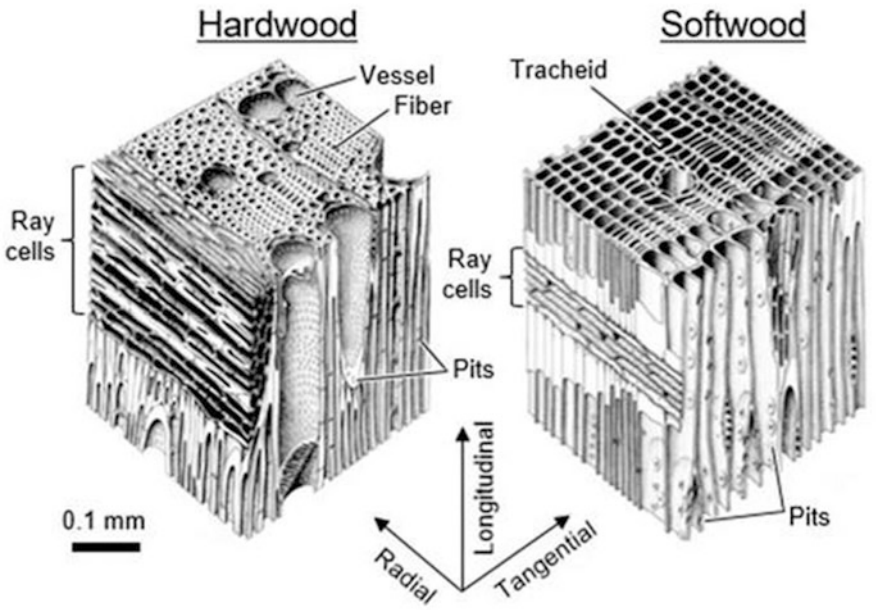


Fig. 20.3 Wood cell wall structure: middle lamella (ML), primary wall (P), secondary wall (S1, S2, S3); reprinted [59]

stage parenchyma decomposition, and a resistance from libriform fibres to white-rot decay, has been confirmed experimentally [14].

The structure of the wood cell wall composing the xylem divides between the middle lamella (ML), primary wall (P) and three layers of secondary wall (S1, S2, S3). They are represented with their fibre orientation and indicative thicknesses in Fig. 20.3, reprinted [59]. The structuring middle lamella is composed of lignin, calcium and pectic compounds that are amorphous. Crystalline cellulose is most prominent in younger wood, so it dominates the S3, and is found in lowering percentages moving towards the P. Inversely, hemicellulose can be found to a greater extent in the P and in reducing shares towards the S3, while lignin is quite evenly spread across the cell wall, with higher concentrations in the ML. The distribution of lignocellulosic compounds in woods is presented in Table 20.3. Colonisation happens through pit apertures present in wood, or the inducing of boreholes by enzymatic activity. Although pectin contents are marginal in wood, pectinases expression is critical to decomposing the pit membrane (Fig. 20.3), which also contains cellulose and allows the fungus to proceed in colonising [61]. Calcium is associated with pectin in wood; Ca^{2+} can combine with fungal oxalic acid and result in pectic acid which solubilises pectin [52]. Calcium, potassium, phosphorus and magnesium are the major elements associated with plant fibre. The mineral content (ash) in softwood is 0.02–1 wt% and 0–5 wt% in hardwood [62]. We can note that large concentrations of calcium oxalate and residues of MnO_2 accumulate during white-rot decay [50]. Moreover, wood can contain a variety of toxic

Table 20.3 Approximated lignin distribution in the different layers of the wood cell wall; adapted [60]

Wood type	Cell type	Layer	Share of total lignin content (%)	Lignin concentration (%)
Gymnospermous early wood	Tracheid	S1–S3	65	24
		ML, P	21	49
		Cell corners	14	64
Gymnospermous late wood	Tracheid	S1–S3	75	22
		ML, P	14	51
		Cell corners	11	78
Angiospermous wood	Fibre	S1–S2	60	19
	Fibre	ML, P	9	40
	Fibre	Cell corners	9	85
	Vessel	S1–S3	9	25
	Vessel	ML, P	2	40
	Ray cells	S1–S3	11	25

compounds, mainly polyphenols or tannins in angiospermous trees and phenolic compounds in gymnosperms. Selective delignification-capable fungi can degrade polyphenols. *Ganoderma* spp. are able to degrade polyphenolic compounds and show chemotropic growth towards these [63]. Nitrogen availability is critical to fungi for enzyme synthesis; the limited amounts in wood makes it resilient to decay (the C:N ratio of birch wood is typically of 55 and 401 in sycamore wood). This compound is mostly found in parenchyma and is known to migrate towards the bark surface during log desiccation [64]. Moreover, wood cell walls specialise depending on local plant mechanical stress and taxonomic order (Fig. 20.4, reprinted [65]), which may lead to a more specific consideration of lignocellulosic substrates to establish cultivation protocols.

The wide distribution of wood-decaying fungi suggests, with increasing evidence, that local environmental factors and substrate specifics drive and regulate colony extent between the various fungal species present. Furthermore, the pH of wood is most often in the 4.0–5.5 range (beech: 5.11, birch: 5.29, for instance) [66]. It has been demonstrated that substrate pH is also influenced by the action of decay [46]. It has been reported, by comparative transcriptonics, that white-rot related genes of a *Phanerochaete carnosa* species would all express in fir (*Abies balsamea*), pine (*Pinus contorta*), spruce (*Picea glauca*) and maple (*Acer saccharum*), but to a varied extent. For instance, MnP genes read was 2.42 times higher in fir compared to maple, but LiP-related genes read was 4.7-fold higher in

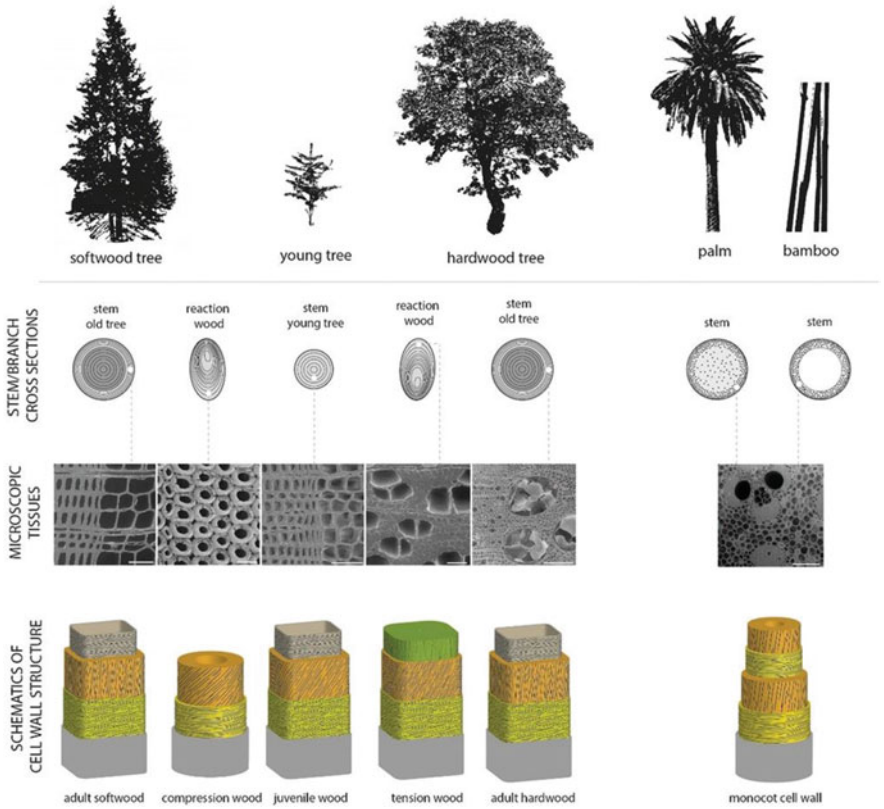


Fig. 20.4 Structure of arboreal plants; reprinted [65]. Microscopic tissues from left to right: adult spruce wood (bar: 50 μm), spruce compression wood (bar: 20 μm), juvenile spruce wood (bar: 50 μm), poplar tension wood (bar: 50 μm), adult oak wood (bar: 200 μm) and vascular bundle of bamboo (bar: 200 μm). Last row showing schematics of cell wall structures. Light grey indicates the compound middle lamella (0.5–1.5 μm) and primary cell wall (approximately 0.1 μm); yellow: S1 layer with a thickness of 0.1–0.35 μm; orange: S2 layer as 1–10 μm thick; inner light grey layer shows the 0.5–1.1 μm thin S3 layer; the green G-layer in tension wood fibres can fill the whole lumen and the cellulose fibrils are oriented along the longitudinal cell axis

maple [67]. This indicates both the resilience of species to the use of a variety of vernacular substrates, but also a precariousness considering the sheer distribution of competitors of an individual species. This can lead to fungi chemically wrestling for resource [68]. *Ganoderma* spp. have been recognised as having the competitive advantage of being able to degrade reaction zones in trees [63]. Studies of lignicolous fungal ecological distribution point to water content and temperature variations being the principal drivers of selection [69]. Climate change has then been related to shifts in fungal niches across hosts [70]. In the wild, host tree species and their evolutionary stage can have an influence on patterns of decay; the tree group that has the most diffuse xylem vessel system may result in a slower initial

colonisation. Similarly, gymnospermous wood having 3–4 mm long tracheids benefits from a cellular compartmentalisation strategy to reduce fungal decay, despite its guaiacyl lignin content. These topological effects are prominent at the early stage of fungal colonisation [64].

20.4 Water in Fungal Decay

The presence of capillary water in the substrate is a prerequisite to allow extracellular transport of metabolites in fungi, and to establish osmotic potential for allowing turgor pressure and subsequent hyphal growth. Turgor also enables fungi to penetrate solid substrates [71]. It has been reported that the radial growth of white-rot species *Physisporinus vitreus* at pH 5 and 25 °C increased from just below 0.5 mm/day at $a_w = 0.97$ to 4.5 mm/day at $a_w = 0.998$ [72]. The production of enzymes by fungi and their catalytic activity has been related to water activity with varying local optima depending on the depressor that was used in the study, pointing to $a_w \geq 0.99$ for optimal catalytic activity here [73].

SSF requiring the absence of solution in between particles for a mycelium to develop, the substrate water holding capacity is an important parameter for ensuring an optimal fungal decay and growth [11, 73, 74]. In timber, moisture can exist as bound water within cell walls below the fibre saturation point (FSP), free liquid water in cell cavities above FSP and water vapour. Relative humidity (RH) of up to 97–98% is generally considered to correspond to cell wall uptake (hygroscopic range), and as RH moves towards saturation, wood cell void uptake tends to become more significant in the moisture distribution (over-hygroscopic range) [75]. The latter is conventionally considered as an optimal range for optimal fungal growth in lignocellulosic SSF. The validity of this convention is nonetheless discussed as it was shown that *T. versicolor* would colonise European beech wood at a minimum moisture content (MC) of 15% for a mass loss over 2% (equivalent to 75–80% RH at −1–37 °C); [76]. Brown-rot fungi grow at 92–97% RH, and that instrumental limitations are encountered in the over-hygroscopic range, thus complicating measures and experimental control [75]. For instance, the FSP of European beech wood has previously been determined to be 24.02% MC at 97% RH and 20 °C [77]. The minimum moisture threshold has been introduced as the metrics having the most significant impact on predicting fungal growth [11, 78]. It represents the MC below which fungal decay cannot be initiated, and is typically given at an mass loss 2%.

Available hydroxyl groups are the predominant sorption sites for water molecules in wood. Their presence in hemicellulose is twice as high as in lignin and four times higher than that of cellulose [79]. It has been reported that cellulose can endure structural changes while wood is desiccated [80, 81]. In the literature, it was hypothesised that lower desiccation rates would give enough time for amorphous cellulose to crystallise, rendering the substrate less hydrophilic. On the contrary, higher rates of desiccation may lead to more prominent amorphous cellulose and more hydroxyl availability. Lignin, hemicellulose and pectin interrupt cellulose

crystallisation, which reflects on the higher crystalline content towards the S3 cell wall layer [82]. Because hemicellulose has a softening point at room temperature and 18–20% MC [74], while lignin softens above 60 °C at a minimum of 15% MC [83], and cellulose is hardly softened in its crystalline conzuration (200 °C), higher thermal treatments may result in hemicellulose degradation, thus rendering the substrate less hydrophilic [84].

Finally, the amount and quality of water in the SSF system varies during fermentation. Fungal oxidative decay of polysaccharides frees water along with CO_2:

$$(C_6H_{10}O_5)_n + 6n(O_2) = 6n(CO_2) + 5n(H_2O) \quad (20.1)$$

where $C_6H_{10}O_5$ is the repeating unit of glucose-based polymers, such as found in starch or cellulose. As bound water is also released during decay, and while substrate dry mass loss occurs, the MC increases if the system is closed. The activity and location of water in SSF systems are are then necessary information to predict fungal development. They are of primary interest to design and produce MBC. To the best of our knowledge, the role of water in MBC production has not yet been reported. The optimal moisture content for laccase, LiP and MnP activation is consistently reported to be 60–75% [85–88].

20.5 Regulating Enzymatic Activities

MBC protocols can be adjusted based on the chemical profile of locally available wood resources and selected fungal species to achieve various design goals. This an be approached by pH and temperature regulation, and monosaccharide, nitrogen and metal ion supplementation. Optimal ligninase activity was reported at pH 4.0–5.0 and 30–35 °C [85, 86]. The pH regulation can be achieved by the addition of $CaCO_3$ in the substrate as a buffering agent to avoid acidification from CO_2 production. Gypsum supplementation has been reported in MBC cultivation [89]. While its use has no impact on the substrate pH, it contributes to adding calcium to the medium. This is useful for pectate lyase activity if the wood has a low Ca^{2+} content, which acts on the 1–4% share of pectin in wood. Varying the C:N ratio has also been shown to lead to an optimum of 20:1 [85]. The form of the C- and N-rich additives has been reported to have an impact on specific enzyme activation. Maltose and urea addition was reported to increase LiP and MnP production in a *T. versicolor* species, while glucose and yeast resulted in increased laccase production [87]. A similar investigation with a *G. lucidum* reported that the use of beef extract increased LiP production by approximately 30% as compared to using yeast extract, peptone or urea, and sucrose had a significant effect on ligninase activity as per other sugar types [85]. Wheat bran is a frequently used substrate additive in SSF. It contains high contents of β-glucans, xylan, cellulose, lignin, B vitamins and mostly minerals (magnesium, phosphorus, manganese, zinc and iron) [90]. The success of this supplement [91] may be related to its mineral contents, especially manganese as it

is a limiting compound for MnP and VP activity [92]. Supplementation of substrates by $MnSO_4$ has therefore been shown to result in an increase in ligninase activity in *S. commune* [86] – most probably by synergistic action of enzymes. Biofuel production aims at efficient extraction of sugars from wood. Selective delignification capable fungi have been extensively investigated in this context. The addition of phenols has been reported to be considerably promoting ligninase activity [93]. Syringic acid has been shown to increase LiP and laccase activity, and gallic acid for MnP in the same study. With an optimised substrate, the authors reported increase of 2.4 to 45.5-fold across the diverse ligninase activities, leading to a 2.3-flod improvement in lignin decay and 7.1 in selectivity value (selectivity is expressed as the ratio of lignin degradation over cellulose and hemicellulose degradation) [94]. The idea of highly selectively degrading lignocellulosic substrates is a very promising track for MBC research. The addition of H_2O_2 to the substrate, necessary for ligninase activity, has also been investigated with significant results [95, 96]. $CuSO_4$ supplements have been shown to increase laccase activity [93]. The use of surfactants such as Tween-80 has been reported to increase ligninase permeation through the cell wall [97], and enhance the solubilisation of lignin and improve hydrolytic activities [98], and even proved useful more recently in promoting all ligninase activity, at a 4 wt% optimum for *G. lucidum* fermentation [88]. With an approximate 80% content of starch, 12% proteins, 3% of other monosaccharides (arabinose, xylose, galactose, glucose) and traces of ash (minerals) [99], white and whole wheat flour constitute useful additives to promote cellulase and hemicellulase activities. Moreover, the use of spent coffee ground is a widespread decay promoter in MBC craft. It is composed of 39% hemicellulose (predominantly mannose and galactose), 24% lignin, 17% proteins, 2% fat and 1.3% ashes. Although it has a high lignin content, coffee grounds contribute to increasing nitrogen and mineral content in the substrate, additionally to glucose polymers. Coffee silverskin, a by-product of the roasting industry, has a quite different chemical profile: 24% cellulose, 29% lignin, 19% proteins, 17% hemicellulose (primarily xylose) and a remarkable 5% ashes [100]. Phenolic compounds present in both substrates have been investigated for their antifungal abilities against white-rot fungi. Using a *T. versicolor* species, it was reported that the supplementation of 3% coffee silverskin extract resulted in about 65% growth inhibition, while 1% caffeine led to a complete inhibition and 1% spent coffee ground in about 20% inhibition [101]. This is related to the introduction of phenolic antioxidants such as caffeine, caffeoylquinic acid lactone and feruoylquinic acid [102–104]. Furthermore, laccase treatment of lignocellulosic substrates has been shown to increase lignin antioxidant activity [105]. Finally, the addition of phosphorus and calcium in the form of superphosphate ($CaH_6O_8P^{2+}$) has proved to increase decay yield. Varying the supplementation from 0.025 wt% to a 0.3 wt% optimum in wheat straw, lignin degradation increased by a 4.5-fold, while cellulose, hemicellulose and protein degradation increased by a factor of about 2.4, while enduring *P. ostreatus* decay [106]. A higher optimum of 0.5 wt% superphosphate addition has been reported for alkaliphilic white-rot *Coprinus* spp. [107], and even 1.0 wt% with *T. versicolor* [108].

20.6 Mycelium Mechanical Properties

Mycelium acts as a binder between wood particles. It does so by degrading lignocellulosic compounds and producing a dense hyphal network that interlocks dispersed particles. The binding between two particles is not distinctly strong, but the very high redundancy in the composite can result in a mechanically valuable material. It has been reported that a *P. ostreatus* mycelium would be more than two times stiffer than a *G. lucidum* one when both cultivated on a cellulose medium [109]. Authors comment on this difference with regard to the higher protein and lipid content in *G. lucidum* that can act as a plasticiser. Furthermore, in this study a set of experiments grown on a PDB-cellulose medium has been compared to the previously mentioned results, and it was found to decrease the Young's modulus of the *G. lucidum* mycelium film by a factor of 3 while increasing its elongation at break by 2.3-fold. This corroborates their previous comment on plasticisers, and suggests that nutrition medium tuning can help composite behaviour. This study suggests that the matrix phase of the composite can be made closer to an elastomer or stiffened by varying protein, lipid or alcohol contents. Similar findings have been reported [111]. Isolated mycelium mechanical behaviour has been investigated in order to model it [112]. This referenced study relies on the characterisation in tension and compression of a pure mycelium. At the resolution of hyphae, researchers have identified three main stages of response of a ligninolytic fungal mycelium: linear elastic behaviour at small strains (hyphae bending); fibre buckling and local structural collapse or densification at larger strains; rapid stiffening associated with full compaction and large number of inter-fibre contacts. To fit their finite-element analysis simulation model, a hyperelastic model was used in this study as mycelium undergoes large displacements in its elastic behaviour and results in a non-linear stress-strain response. Young's moduli are situated in the 0.6–2.0 MPa range for a density in 30–50 kg/m^3, the yield stress in 40–80 kPa and ultimate tensile strength in 100–300 kPa [110]. The results are presented in Table 20.4.

Table 20.4 Tensile behaviour of mycelia from *P. ostreatus* (PO) [109], *G. lucidum* (GL) [109] and an undisclosed species (ND) [110]

Tensile behaviour	PO[a]	PO[b]	GL[a]	GL[b]	ND[c]
Young's modulus (MPa)	28	17	12	4	0.6–2.0
Ultimate strength (MPa)	0.7	1.1	1.1	0.8	0.1–0.3
Elongation at break (%)	4	9	14	33	–

[a] Cellulose medium.
[b] Cellulose and PDB medium.
[c] Undisclosed medium.

20.7 Substrate Mechanical Properties

Most commercially valuable woods have a Young's modulus of 5.5–15.7 GPa at 12% MC, and 4.4–12.3 GPa when freshly cut (green) [113]. The distribution of these performances is similar in gymnospermous and angiospermous woods. Wood particles are therefore important load-carrying members of the composite system as compared to mycelia elasticity discussed previously, and reduce the magnitude of stress experienced by the mycelial matrix. The angular shapes of wood debris contribute to crack initiation and the dewetting behaviour of the larger particles is a principal contributor to damage nucleation in such a two-phase particulate composite, although they contribute to composite stiffening by matrix shielding. Furthermore, the shape, nature and distribution of particles in two-phase particulate composites has been shown to have a substantial influence over the load transfer between members, and hence their overall stiffness [114]. Moreover, while lignin is a primary contributor of strength parallel to grain, hemicellulose supports compression strength perpendicular to grain. Its decay greatly affects the structural integrity of wood and its hardness [115].

In particle studies of granular materials, shape parameters such as flakiness/flatness (ratio of particle thickness to width), elongation (length-to-width ratio), sphericity (deviation from a sphere geometry) and roundness/angularity (measure of angular sharpness) have been investigated [116]. Without matrix phase (the stiffest reported pure mycelium of a *P. ostreatus* strain in 20.6 had a Young's modulus of 28 MPa, 178–534 times more elastic than woods), studies have reported that a 3:1 ratio of flaky to bulky particle content would be an approximate optimal for shear strength (depending on the system of study). This was related to cohesion increase under stress due to particle interlocking. Furthermore, it was reported that a higher particle angularity leads to a decrease in elastic modulus, while ultimate strength increases. The shear strength was reported to follow the same dynamic, increasing with particle angularity. Increasing flakiness and angularity leads to increased cohesion and subjection to higher abrasion. This leads to damage accumulation under repeated loads, thus leading to strain accumulation [116].

While wood particles are of common use in MBC, other more elastic substrates have been used such as nonwoven cotton fibres [117]. A few studies have investigated the addition of non-organic aggregates to a lignocellulosic substrate to improve its stiffness, such as with carbonate sand [118] (which also acts as a pH buffer being mainly composed of $CaCO_3$) and sand and gravel [9]. It was reported that supplementation of a cotton and wheat bran medium with 37.5 wt% carbonate sand resulted in a 1.6-fold stiffening, and a factor 4 increase in ultimate compressive strength, while density increased by 27%.

20.8 Composite Mechanical Properties

The large number of influential variables on MBC mechanical properties leads to the emergence of a variety of methods for tuning them. While common practice leads to an elastic modulus of an approximate maximum of 2 MPa, post-cultivation heat-pressing has been reported to lead to moduli in the range of 35–97 MPa, and flexural moduli in the range of 34–80 MPa [117]. The use of particle-additive strategies, additionally to a wheat bran supplemented medium made of cotton stalk, has proven to have a significant effect: an elastic modulus of 48.5 MPa was reported with 37.5% carbonate sand addition with *P. ostreatus*, while a non-supplemented control in this study reached 30.3 MPa [118]. A second study reported an elastic modulus situated in the 39–60 MPa range for a density in 240–265 kg/m^3 [7]. Authors of the latter are elusive about production details, but nonetheless indicate the use of Alaska birch shavings (*Betula neoalaskana*), millet grain, wheat bran, calcium sulphate and the addition of a natural fibre. The specifics of the performative batch are not disclosed, but a pre-colonisation was performed prior to moulding. The performance increase does not come from a single factor considering the experimental plan. The considerable use of fibres (of undisclosed quality), grains, brans and $CaSO_4$ leads us to understand this as a hybrid improvement using both substrate-based reinforcement with the fibres and additives. Other strategies have been investigated, such as with the introduction of SBR Latex, a bonding agent for the construction industry. With a 5% addition of SBR Latex and 0.5% silane coupling agent to a cotton seed hull medium, the cultivation of a *P. ostreatus* species displayed a 2.39 factor increase in ultimate strength with a 27.6% density increase [119]. The resulting environmental impact has not been discussed in the study. The addition of another material with high environmental impact [120], cellulose nanofibrils (CNF), has been reported [121]. Between two specimen groups, the optimal supplementation of CNF was reportedly 2.5–5 wt%, with a contribution in Young's modulus of a factor 2.56–5.6 and 2.8–7 in ultimate strength. In this study, the effect of densification on composites embedding a 2.5 wt% CNF part was reported: densifying from 300 to 600 kg/m^3 contributed to increase the yield strength exponentially to a factor 17, while the stiffness increased 14.38-fold.

An extensive study of the properties in tension of densified MBC reported that elastic moduli situated in a range of 3.0–13.0 MPa; these values resulting in 24 days cultivated *T. versicolor* mycelia on wheat bran supplemented beech sawdust [117]. For this specimen group, a flexural modulus of 9.0 MPa was reported. The state-of-the-art in MBC research largely considers monolithic and homogeneous composites, besides a few study groups investigating jute type materials in sandwich composite reinforcement and composition with wood panels [45, 122–124].

Across the state-of-the-art, we can identify three main research strategies: *densification* (by composite packing, cold- or hot-pressing), *composition* (by introducing structuring elements such as fibres and aggregates) and *supplementation* (by chemical tuning of the substrate). These are substrate-focused strategies, targeting mycelium properties tuning in the case of supplementation, while the very choice of the fungal species can also result in radically different cultivation lead time and

mechanical properties. Genetic modification of fungi could promote specific enzymatic activity or modify the composition of its cell wall to help improve performance. However, the investment cost and ethics of this practice may lead to prioritising more energy-efficient and affordable techniques.

20.9 Analysis of the State-of-the-Art

So as to analyse the effect of strategies in the MBC state-of-the-art and to present a comprehensive mechanical review for it, we collected reports of elastic moduli and ultimate strength in compression, tension and flexion, along with principal substrate information, species and density [7, 45, 117, 118, 121, 122, 124–135]. There are 105 data points gathered from 18 journal and conference articles. These include articles reporting on all or some of these metrics: strength and/or stiffness in compression, tension and/or flexion; 19 data points had no density reported. In the following data visualisations (Figs. 20.5, 20.6, 20.7, 20.8, and 20.9), only the reports with sufficient data are rendered.

The distribution of species to substrates reports is heterogeneous. For instance, we can observe in Fig. 20.5 that no peer-reviewed report has been made of hemp cultivated *G. lucidum* as per mechanical behaviour. This figure does not consider all substrates and supplements included in MBC, only the primary one.

We present the specific moduli and strengths as per substrates in Fig. 20.6, and specific moduli and strengths as per species in Fig. 20.7. Larger marker size indicates

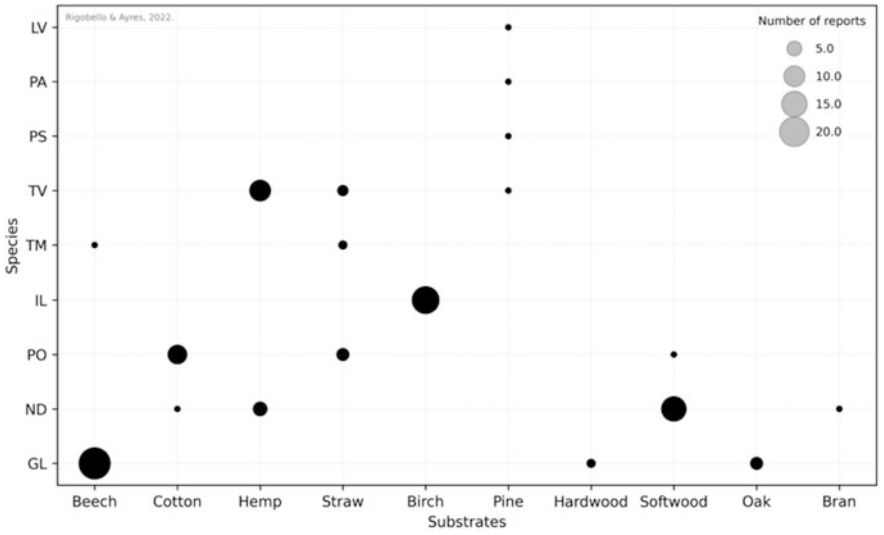


Fig. 20.5 Reports of principal substrates and species. GL: *Ganoderma lucidum*, ND: undisclosed, PO: *Pleurotus ostreatus*, IL: *Irpex lacteus*, TM: *Trametes multicolor*, TV: *Trametes versicolor*, PS: *Pycnoporus sanguineus*, PA: *Pleurotus albidus*, LV: *Lentinus velutinus*

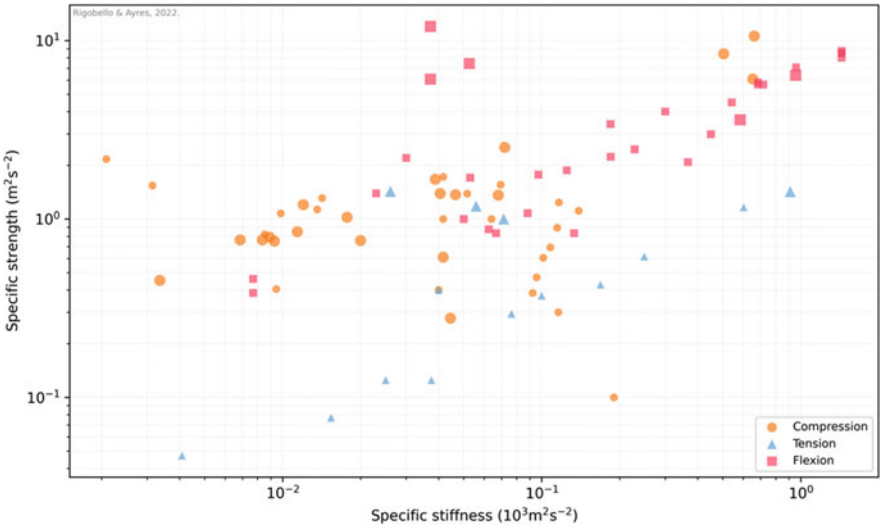


Fig. 20.6 Specific strength and stiffness per substrate. Larger markers correspond to composition strategies

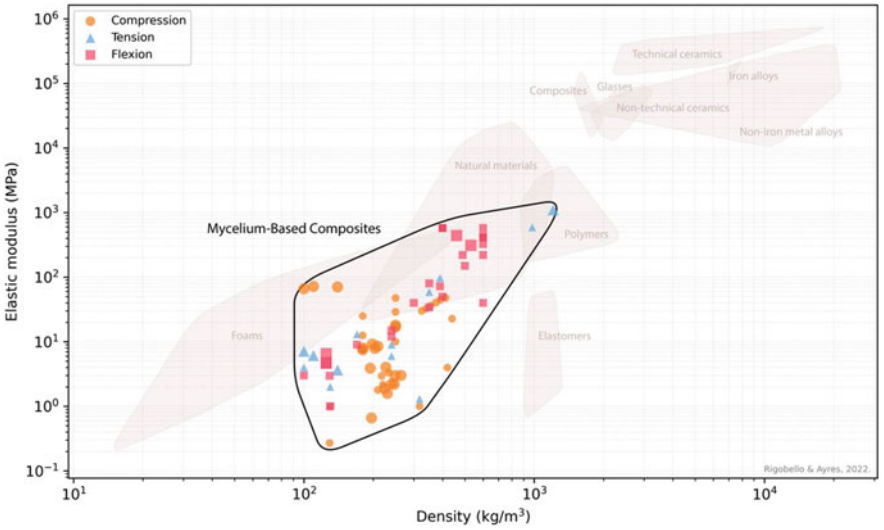


Fig. 20.7 Specific strength and stiffness per species. Larger markers correspond to composition strategies

a composition strategy. We classified MBC having structuring fibres, textiles, inorganic particles, CNF and long fibre balls. Only a few reports on MBC tension modulus (14 data points) and strength (15 data points) have been published. From biochemical review presented above, we can be surprised by the mechanical

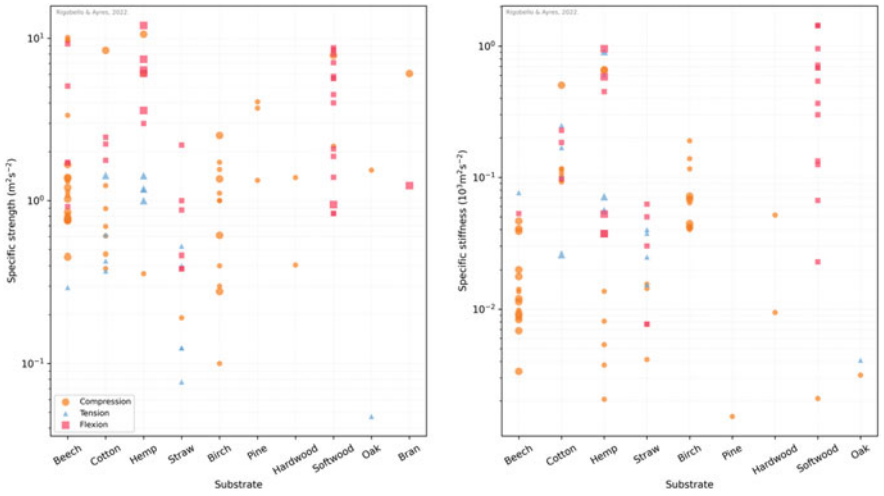


Fig. 20.8 Specific strength as a function of specific stiffness. Larger markers correspond to composition strategies

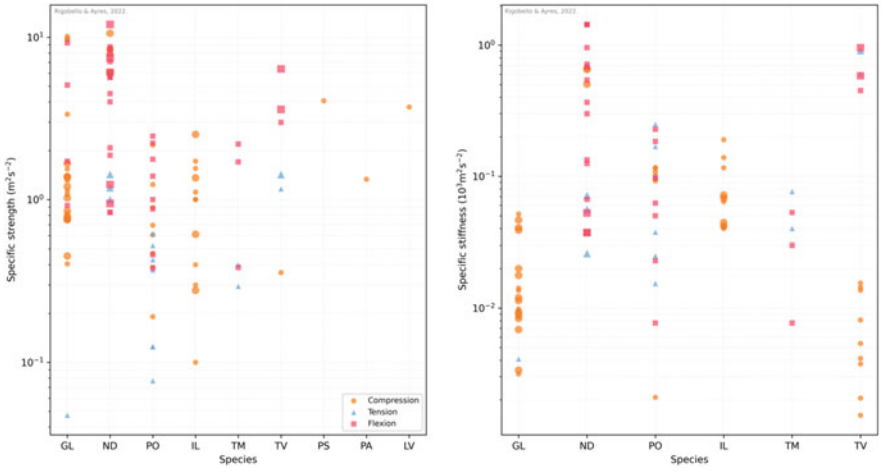


Fig. 20.9 Elastic modulus as a function of density. Larger markers correspond to composition strategies

competitiveness of softwood in Fig. 20.6. It should be noted that these specific reports are of CNF-embedding MBC, thus supplementing softwood with cellulose that is accessible to the fungus without a lignin barrier, having been pressed at an undisclosed pressure at 180 °C for 15 min [121]. Densification of lignocellulosic particles by hot pressing may result in an increased mechanical interlocking of the particles, along with chemical binding from solubilised compounds [83]. Furthermore, in Fig. 20.7, we can identify the undisclosed species in use to be the most

performative with regards to specific stiffness and specific strength. In Fig. 20.8, we present the specific strength as a function of specific stiffness in compression, tension and flexion. We can notice in this figure a departure from the state-of-the-art of a specific study in compression [124]. The specimens of the latter were embedding a surface-binding fabric and packed cotton-ginning waste and hemp pith of undisclosed qualities. The reported specimen densities are of 100–140 kg/m^3. Without further details on the cultivation method, and with the tests having been conducted with only 3–4 replicates, we can hardly conclude on the causes having led to these outstanding values. With critical methodological details being undisclosed, this study is irreproducible and, as such, serves solely as advertisement material.

We present an Ashby map of state-of-the-art MBC data for elastic modulus as a function of density in Fig. 20.9. We can notice that MBC represent a large functional area, mostly situating in between foam and elastomer materials, with reports extending the group span to performances typical of natural materials – the upper-right cluster of flexural modulus reports values typically stiffer than cork and leather. Punctual reports of tensile modulus in the upper-right MBC area would be of a similar stiffness than polyethylene (PE) or PE polymers. The span of the MBC area reflects the three development strategies: densification leads to clear tendency towards increasing the stiffness and strength of MBC for construction materials; supplementation is less distinct at the moment in the reports, but the three data points in the upper-left MBC area are representative of its effect as shown in Fig. 20.8; composition strategies are distributed across the MBC area and reflect a wide variety of inserts and resulting mechanical behaviour. Composition has been demonstrated to lead to significant effect on MBC behaviour in failure control or stiffening for instance [45].

20.10 Conclusions

This review focuses on three aspects: fungal identity (fungal species enzyme array), nutrients (substrate chemical profile and wetting characteristics) and substrate architecture (substrate aggregate characteristics, density and composition). From the enzyme review, we have come to understand that the substrate fitness and mechanical behaviour of mycelia can be adjusted by supplementation. This can be achieved by pH and temperature regulation, and monosaccharide, nitrogen, metal ion and $CaCO_3$ supplements could be systematically investigated in the future as per initial substrate pH and its variation during fermentation. Lignocellulosic substrate selection can also have a significant effect, with non-supplemented softwoods being chemically unfit for optimal white-rot decay. The use of sycamore or ash wood could be investigated in the future as they display higher syringyl monomer contents. The C:N content of sycamore being 401, supplementation may be necessary for optimal fermentation. Furthermore, incubation temperature is commonly fixed throughout the fermentation process below 30 °C, which promotes specific enzymatic activities (LiP, MnP, DyP, xylanase, pectate lyase, GH1 β-glucosidase and

GH3 β-glucosidase). While investigating exothermic heat generation and dynamic temperature control related to species, genome distribution may lead to both a widening and deepening of the available material qualities and versatility.

The effect of water activity upon MBC colonisation-related performances, such as mechanical response, has not yet been reported in the state-of-the-art. Considering reports in SSF studies of several fold increase in colonisation rates, investigating the effect of water activity in MBC may result in longer production times and performance. Moreover, qualifying the effect of hydroxyl group availability in hemicellulose and cellulose, depending on wood processing history, could lead to further medium optimisation. We expect this particular aspect to be specifically useful for paste-like substrate design, such as that found in mycelium-related 3D printing.

Of course, stimulating the focus of future research towards such specific aspects may result in a higher entry barrier for a technique that has a strong interest for its affordability. The use of Fourier transform IR or other spectrometric process by experts within a network of amateur practitioners could be useful to adapt cultivation strategies constructively to local contexts; the qualification of an available substrate may lead to specifying necessary processing, supplementation, species selection and/or relevant composition strategies. While industrial and academic research may result in defining a panel of protocols for the selection of prospective materials, situated citizen craft practices based on locally available materials could coexist.

Across the MBC state-of-the-art, we have identified three principal substrate-oriented design strategies: densification, supplementation and composition. The latter covers a variety of strategies, from CNF introduction to uniaxial reinforcement. These will need to be further investigated to allow an in-depth understanding and serving a goal-oriented material development. It is important to note that most experimental methods are heterogeneous, may not be statistically significant or only partially disclosed. A structured approach to developing material designs along with assessing them will most likely result in the concentration of the state-of-the-art around functional poles in the future, and will help to drive material development in depth. Additionally, future reproducibility studies and critical methodological reviews of MBC studies may both improve the overall market readiness and public adoption of MBC solutions. This can further valorise the wealth of material, design and semiotic properties that the versatility and affordability of MBC systems support.

In this chapter, we have identified the principles driving MBC design and engineering, and have attempted to make these comprehensible. Beyond this focus on the design and production, MBC engage broader discourses related to resource sourcing, circular economy and sustainability. Because MBC have a higher vernacular potential [136], they can be made of recycled substrate, or materials locally and seasonally available. MBC also benefit from unique aesthetics as a result of the phylogenic expression of fungi through craft and substrate design [137]. We hope that the on-going uncovering of the principles for predicting MBC performance contributes to an engineering practice that may satisfy industrial needs (which detrimentally often actualises in patents [138]), but also fosters a rich, open, sustainable and creative citizen craft.

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References

1. Duggar BM (1905) The principles of mushroom growing and mushroom spawn making. U.-S. Government Printing Office
2. Attias N et al (2020) Mycelium bio-composites in industrial design and architecture: comparative review and experimental analysis. J Clean Prod 246:119037. https://doi.org/10.1016/j.jclepro.2019.119037
3. Appels FVW, van den Brandhof JG, Dijksterhuis J, de Kort GW, Wösten HAB (2020) Fungal mycelium classified in different material families based on glycerol treatment. Commun Biol 3(1):1–5. https://doi.org/10.1038/s42003-020-1064-4
4. Ohm RA et al (2010) Genome sequence of the model mushroom Schizophyllum commune. Nat Biotechnol 28(9):957–963. https://doi.org/10.1038/nbt.1643
5. Sawitzke JA et al (2013) Recombineering: using drug cassettes to knock out genes in vivo. Methods Enzymol. Laboratory Methods in Enzymology: Cell, Lipid and Carbohydrate 533: 79–102. https://doi.org/10.1016/B978-0-12-420,067-8.00007-6
6. Alves AMCR et al (2004) Highly efficient production of laccase by the basidiomycete pycnoporus cinnabarinus. Appl Environ Microbiol 70(11):6379–6384. https://doi.org/10.1128/AEM.70.11.6379-6384.2004
7. Yang Z, Zhang F, Still B, White M, Amstislavski P (2017) Physical and mechanical properties of fungal mycelium-based biofoam. J Mater Civ Eng 29:04017030. https://doi.org/10.1061/(ASCE)MT.1943-5533.0001866
8. Tacer-Caba Z, Varis JJ, Lankinen P, Mikkonen KS (2020) Comparison of novel fungal mycelia strains and sustainable growth substrates to produce humidity-resistant biocomposites. Mater Des 192:108728. https://doi.org/10.1016/j.matdes.2020.108728
9. Moser FJ et al (2017) Fungal mycelium as a building material. In: The annual symposium of the International Association for shell and spatial structures. Fachgruppe Biologie, Hamburg
10. Chen S et al (2012) Genome sequence of the model medicinal mushroom Ganoderma lucidum. Nat Commun 3(1):913. https://doi.org/10.1038/ncomms1923
11. Brischke C, Alfredsen G (2020) Wood-water relationships and their role for wood susceptibility to fungal decay. Appl Microbiol Biotechnol 104(9):3781–3795. https://doi.org/10.1007/s00253-020-10479-1
12. Floudas D et al (2012) The paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. Science 336(6089):1715–1719. https://doi.org/10.1126/science.1221748
13. Castaño JD, Zhang J, Anderson CE, Schilling JS (2018) Oxidative damage control during decay of wood by brown rot fungus using oxygen radicals. Appl Environ Microbiol 84(22): e01937–e01918. https://doi.org/10.1128/AEM.01937-18
14. Schwarze FWMR (2007) Wood decay under the microscope. Fungal Biol Rev 21(4):133–170. https://doi.org/10.1016/j.fbr.2007.09.001

15. Tinoco R, Verdin J, Vazquez-Duhalt R (2007) Role of oxidizing mediators and tryptophan 172 in the decoloration of industrial dyes by the versatile peroxidase from Bjerkandera adusta. J Mol Catal B Enzym 46(1):1–7. https://doi.org/10.1016/j.molcatb.2007.01.006
16. Zamocky M et al (2006) Cellobiose dehydrogenase–a flavocytochrome from wood-degrading, phytopathogenic and saprotropic fungi. Curr Protein Pept Sci 7(3):255–280. https://doi.org/10.2174/138920306777452367
17. Kubicek CP (2012) Fungi and lignocellulosic biomass. Biomass and biofuels. Wiley-Blackwell, Ames
18. Gomes I, Gomes J, Steiner W, Esterbauer H (1992) Production of cellulase and xylanase by a wild strain of Trichoderma viride. Appl Microbiol Biotechnol 36(5):701–707. https://doi.org/10.1007/BF00183253
19. Paramjeet S, Manasa P, Korrapati N (2018) Biofuels: production of fungal-mediated ligninolytic enzymes and the modes of bioprocesses utilizing agro-based residues. Biocatal Agric Biotechnol 14:57–71. https://doi.org/10.1016/j.bcab.2018.02.007
20. Vyas BRM, Volc J, Šašek V (1994) Effects of temperature on the production of manganese peroxidase and lignin peroxidase by phanerochaete chrysosporium. Folia Microbiol 39(1):19–22. https://doi.org/10.1007/BF02814523
21. Jiang F, Kongsaeree P, Schilke K, Lajoie C, Kelly C (2008) Effects of pH and temperature on recombinant manganese peroxidase production and stability. Appl Biochem Biotechnol 146(1):15–27. https://doi.org/10.1007/s12010-007-8039-5
22. Ravichandran A, Rao RG, Maheswarappa Gopinath S, Sridhar M (2021) Augmenting versatile peroxidase production from Lentinus squarrosulus and its role in enhancing ruminant feed. Bioresources 16(1):1600–1615. https://doi.org/10.15376/biores.16.1.1600-1615
23. Kwang-Soo S, Hwan KY, Jong-Soon L (2005) Purification and characterization of manganese peroxidase of the white-rot fungus Irpex lacteus. J Microbiol 43(6):503–509
24. Rajhans G, Sen S, Barik A, Raut S (2020) Elucidation of fungal dye-decolourizing peroxidase (DyP) and ligninolytic enzyme activities in decolourization and mineralization of azo dyes. J Appl Microbiol 129(6):1633–1643. https://doi.org/10.1111/jam.14731
25. Desai S et al (2011) Isolation of laccase producing fungi and partial characterization of laccase. Biotechnol Bioinformatics Bioeng 1(4):543–549
26. Rashmi R, Siddalingamurthy KR (2018) Microbial xyloglucanases: a comprehensive review. Biocatalysis Biotransformation 36(4):280–295. https://doi.org/10.1080/10242422.2017.1417394
27. Motta FL, Andrade CCP, Santana MHA (2013) A review of xylanase production by the fermentation of xylan: classification, characterization and applications. In: Chandel AK, da Silva SS (eds) Sustainable degradation of lignocellulosic biomass – techniques, applications and commercialization. IntechOpen. https://doi.org/10.5772/53544
28. Moreira LRS, Filho EXF (2008) An overview of mannan structure and mannan-degrading enzyme systems. Appl Microbiol Biotechnol 79(2):165–178. https://doi.org/10.1007/s00253-008-1423-4
29. Yadav S, Yadav PK, Yadav D, Yadav KDS (2009) Purification and characterization of pectin lyase produced by aspergillus terricola and its application in retting of natural fibers. Appl Biochem Biotechnol 159(1):270–283. https://doi.org/10.1007/s12010-008-8471-1
30. Wu P, Yang S, Zhan Z, Zhang G (2020) Origins and features of pectate lyases and their applications in industry. Appl Microbiol Biotechnol 104(17):7247–7260. https://doi.org/10.1007/s00253-020-10769-8
31. Cameron MD, Aust SD (2001) Cellobiose dehydrogenase–an extracellular fungal flavocytochrome. Enzym Microb Technol 28(2):129–138. https://doi.org/10.1016/S0141-0229(00)00307-0
32. Nataraja S, Chetan DM, Krishnappa M (2010) Effect of temperature on cellulase enzyme activity in crude extracts isolated from solid wastes microbes. Int J Microbiol Res 2:44–47. https://doi.org/10.9735/0975-5276.2.2.44-47

33. Heinzelman P et al (2009) A family of thermostable fungal cellulases created by structure-guided recombination. Proc Natl Acad Sci 106(14):5610–5615. https://doi.org/10.1073/pnas.0901417106
34. de Cássia Teixeira da Silva V et al (2016) Effect of pH, temperature, and chemicals on the endoglucanases and β-glucosidases from the thermophilic fungus myceliophthora heterothallica F.2.1.4. Obtained by solid-state and submerged cultivation. Biochem Res Int 2016:e9781216. https://doi.org/10.1155/2016/9781216
35. Hofrichter M, Ullrich R, Pecyna MJ, Liers C, Lundell T (2010) New and classic families of secreted fungal heme peroxidases. Appl Microbiol Biotechnol 87(3):871–897. https://doi.org/10.1007/s00253-010-2633-0
36. Watanabe T, Shirai N, Okada H, Honda Y, Kuwahara M (2001) Production and chemiluminescent free radical reactions of glyoxal in lipid peroxidation of linoleic acid by the ligninolytic enzyme, manganese peroxidase. Eur J Biochem 268(23):6114–6122. https://doi.org/10.1046/j.0014-2956.2001.02557.x
37. Lundell TK, Mäkelä MR, Hildén K (2010) Lignin-modifying enzymes in filamentous basidiomycetes – ecological, functional and phylogenetic review. J Basic Microbiol 50(1):5–20. https://doi.org/10.1002/jobm.200900338
38. Baldrian P (2006) Fungal laccases – occurrence and properties. FEMS Microbiol Rev 30(2):215–242. https://doi.org/10.1111/j.1574-4976.2005.00010.x
39. Eggert C, Temp U, Eriksson KE (1996) The ligninolytic system of the white rot fungus Pycnoporus cinnabarinus: purification and characterization of the laccase. Appl Environ Microbiol 62(4):1151–1158. https://doi.org/10.1128/aem.62.4.1151-1158.1996
40. Hrmova M, MacGregor EA, Biely P, Stewart RJ, Fincher GB (1998) Substrate binding and catalytic mechanism of a barley beta-D-glucosidase/(1,4)-beta-D-glucan exohydrolase. J Biol Chem 273(18):11134–11143. https://doi.org/10.1074/jbc.273.18.11134
41. Harris PV et al (2010) Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: structure and function of a large, enigmatic family. Biochemistry 49(15):3305–3316. https://doi.org/10.1021/bi100009p.20230050
42. Couturier M et al (2018) Lytic xylan oxidases from wood-decay fungi unlock biomass degradation. Nat Chem Biol 14(3):306–310. https://doi.org/10.1038/nchembio.2558
43. Sista Kameshwar AK, Qin W (2018) Comparative study of genome-wide plant biomass-degrading CAZymes in white rot, brown rot and soft rot fungi. Mycology 9(2):93–105. https://doi.org/10.1080/21501203.2017.1419296.30123665
44. Zhou S et al (2018) Investigation of lignocellulolytic enzymes during different growth phases of Ganoderma lucidum strain G0119 using genomic, transcriptomic and secretomic analyses. PLoS One 13(5):e0198404. https://doi.org/10.1371/journal.pone.0198404
45. Rigobello A, Ayres P (2022) Compressive behaviour of anisotropic mycelium-based composites. Sci Rep 12:6846. https://doi.org/10.1038/s41598-022-10930-5
46. Freschet GT, Weedon JT, Aerts R, van Hal JR, Cornelissen JHC (2012) Interspecific differences in wood decay rates: insights from a new short-term method to study long-term wood decomposition. J Ecol 100(1):161–170. https://doi.org/10.1111/j.1365-2745.2011.01896.x
47. Parfitt D, Hunt J, Dockrell D, Rogers HJ, Boddy L (2010) Do all trees carry the seeds of their own destruction? PCR reveals numerous wood decay fungi latently present in sapwood of a wide range of angiosperm trees. Fungal Ecol 3(4):338–346. https://doi.org/10.1016/j.funeco.2010.02.001
48. Schwarze FWMR, Engels J, Mattheck C (2000) Fungal strategies of wood decay in trees. Springer Science & Business Media, Berlin
49. Adaskaveg JE, Gilbertson RL, Dunlap MR (1995) Effects of incubation time and temperature on in vitro selective delignification of silver leaf oak by Ganoderma colossum. Appl Environ Microbiol 61(1):138–144
50. Blanchette RA (1995) Degradation of the lignocellulose complex in wood. Can J Bot 73:999–1010. https://doi.org/10.1139/b95-350

51. Whetten R, Sederoff R (1995) Lignin biosynthesis. Plant Cell 7(7):1001–1013. https://doi.org/10.1105/tpc.7.7.1001
52. Green F, Kuster TA, Highley TL (1996) Pectin degradation during colonization of wood by brown-rot fungi. Recent Res Dev Plant Pathol 1:83–93
53. van der Werf HMG, van der Veen Harsveld JE, Bouma ATM, ten Cate M (1994) Quality of hemp (Cannabis sativa L.) stems as a raw material for paper. Ind Crop Prod 2(3):219–227. https://doi.org/10.1016/0926-6690(94)90039-6
54. Jakes JE, Hunt CG, Zelinka SL, Ciesielski PN, Plaza NZ (2019) Effects of moisture on diffusion in unmodified wood cell walls: a phenomenological polymer science approach. Forests 10(12):1084. https://doi.org/10.3390/f10121084
55. Fengel D, Wegener G (2011) Wood: chemistry, ultrastructure, reactions. Walter de Gruyter, Berlin
56. Schwarze FWMR, Baum S, Fink S (2000) Dual modes of degradation by Fistulina hepatica in xylem cell walls of Quercus robur. Mycol Res 104(7):846–852. https://doi.org/10.1017/S0953756299002063
57. Schwarze FWMR, Baum S, Fink S (2000) Resistance of fibre regions in wood of Acer pseudoplatanus degraded by Armillaria mellea. Mycol Res 104(9):1126–1132. https://doi.org/10.1017/S0953756200002525
58. Ayuso-Fernández I, Rencoret J, Gutiérrez A, Ruiz-Dueñas FJ, Martínez AT (2019) Peroxidase evolution in white-rot fungi follows wood lignin evolution in plants. Proc Natl Acad Sci 116(36):17900–17905. https://doi.org/10.1073/pnas.1905040116
59. Wiedenhoeft A (2010) Structure and function of wood. In: Wood handbook: wood as an engineering material. GTR-190. U.S. Dept. of Agriculture, Forest Service, Forest Products Laboratory, Madison, pp 3.1–3.18
60. Ferraz A, Costa THF, Siqueira G, Milagres AMF (2014) Mapping of cell wall components in lignified biomass as a tool to understand recalcitrance. In: da Silva SS, Chandel AK (eds) Biofuels in Brazil: fundamental aspects, recent developments, and future perspectives. Springer International Publishing, Cham, pp 173–202
61. Schwarze FWMR, Landmesser H (2000) Preferential degradation of pit membranes within tracheids by the basidiomycete physisporinus vitreus. Wood Res Technol 54(5):461–462. https://doi.org/10.1515/HF.2000.077
62. Tsoumis G (2009) Science and technology of wood: structure, properties and utilization. Verlag Kessel, Remagen
63. Schwarze FW, Ferner D (2003) Ganoderma on trees—differentiation of species and studies of invasiveness. Arboric J 27(1):59–77. https://doi.org/10.1080/03071375.2003.9747362
64. Boddy L (1992) Microenvironmental aspects of xylem defenses to wood decay fungi. In: Blanchette RA, Biggs AR (eds) Defense mechanisms of woody plants against fungi, Springer series in wood science. Springer, Berlin, pp 96–132
65. Eder M, Schäffner W, Burgert I, Fratzl P (2021) Wood and the activity of dead tissue. Adv Mater 33(28):2001412. https://doi.org/10.1002/adma.202001412
66. Geffert A, Geffertova J, Dudiak M (2019) Direct method of measuring the pH value of wood. Forests 10(10):852. https://doi.org/10.3390/f10100852
67. MacDonald J et al (2011) Transcriptomic responses of the softwood-degrading white-rot fungus phanerochaete carnosa during growth on coniferous and deciduous wood. Appl Environ Microbiol 77(10):3211–3218. https://doi.org/10.1128/AEM.02490-10
68. Hiscox J, O'Leary J, Boddy L (2018) Fungus wars: basidiomycete battles in wood decay. Stud Mycol. Leading women in fungal biology 89:117–124. https://doi.org/10.1016/j.simyco.2018.02.003
69. Boddy L (1983) Microclimate and moisture dynamics of wood decomposing in terrestrial ecosystems. Soil Biol Biochem 15(2):149–157. https://doi.org/10.1016/0038-0717(83)90096-2
70. Boddy L et al (2014) Climate variation effects on fungal fruiting. Fungal Ecol. Fungi in a changing world: the role of fungi in ecosystem response to global change 10:20–33. https://doi.org/10.1016/j.funeco.2013.10.006

71. Money NP (1995) Turgor pressure and the mechanics of fungal penetration. Can J Bot 73:96–102. https://doi.org/10.1139/b95-231
72. Fuhr MJ, Stührk C, Schubert M, Schwarze FWMR, Herrmann HJ (2011) Study of the combined effect of temperature, pH and water activity on the radial growth rate of the white-rot basidiomycete Physisporinus vitreus by using a hyphal growth model. arXiv 1106: 4521. https://doi.org/10.48550/arXiv.1106.4521
73. Gervais P, Molin P (2003) The role of water in solid-state fermentation. Biochem Eng J. Solid-State Fermentation 13(2):85–101. https://doi.org/10.1016/S1369-703X(02)00122-5
74. Engelund ET, Thygesen LG, Svensson S, Hill CAS (2013) A critical discussion of the physics of wood–water interactions. Wood Sci Technol 47(1):141–161. https://doi.org/10.1007/s00226-012-0514-7
75. Thybring EE, Kymäläinen M, Rautkari L (2018) Moisture in modified wood and its relevance for fungal decay. IForest 11(3):418–422. https://doi.org/10.3832/ifor2406-011
76. Meyer L, Brischke C, Treu A, Larsson-Brelid P (2016) Critical moisture conditions for fungal decay of modified wood by basidiomycetes as detected by pile tests. Holzforschung 70(4): 331–339. https://doi.org/10.1515/hf-2015-0046
77. Jankowska A, Kozakiewicz P (2016) Determination of fibre saturation point of selected tropical wood species using different methods. Drewno: prace naukowe, doniesienia, komunikaty 59:197. https://doi.org/10.12841/wood.1644-3985.C07.12
78. Brischke C, Soetbeer A, Meyer-Veltrup L (2017) The minimum moisture threshold for wood decay by basidiomycetes revisited. A review and modified pile experiments with Norway spruce and European beech decayed by Coniophora puteana and Trametes versicolor. Holzforschung 71(11):893–903. https://doi.org/10.1515/hf-2017-0051
79. Thybring EE, Thygesen LG, Burgert I (2017) Hydroxyl accessibility in wood cell walls as affected by drying and re-wetting procedures. Cellulose 24(6):2375–2384. https://doi.org/10.1007/s10570-017-1278-x
80. Atalla RH, Brady JW, Matthews JF, Ding SY, Himmel ME (2009) Structures of plant cell wall celluloses. In: Biomass recalcitrance: deconstructing the plant cell wall for bioenergy, pp 188–212
81. Leppänen K et al (2011) X-ray scattering and microtomography study on the structural changes of never-dried silver birch, European aspen and hybrid aspen during drying. Holzforschung 65(6):865–873. https://doi.org/10.1515/HF.2011.108
82. Salmén L (2015) Wood morphology and properties from molecular perspectives. Ann For Sci 72(6):679–684. https://doi.org/10.1007/s13595-014-0403-3
83. Hubbe MA, Pizzi A, Zhang H, Halis R (2018) Critical links governing performance of self-binding and natural binders for hot-pressed reconstituted lignocellulosic board without added formaldehyde: a review. Bioresources 13(1):2049–2115
84. Ramiah MV (1970) Thermogravimetric and differential thermal analysis of cellulose, hemicellulose, and lignin. J Appl Polym Sci 14(5):1323–1337. https://doi.org/10.1002/app.1970.070140518
85. Hariharan S, Nambisan P (2013) Optimization of lignin peroxidase, manganese peroxidase, and lac production from ganoderma lucidum under solid state fermentation of pineapple leaf. Bioresources 8(1):250–271
86. Irshad M, Asgher M (2011) Production and optimization of ligninolytic enzymes by white rot fungus Schizophyllum commune IBL-06 in solid state medium banana stalks. Afr J Biotechnol 10(79):18234–18242. https://doi.org/10.4314/ajb.v10i79
87. Iqbal HMN, Asgher M, Bhatti HN (2011) Optimization of physical and nutritional factors for synthesis of lignin degrading enzymes by a novel strain of Trametes versicolor. Bioresources 6(2):1273–1287
88. Asgher M, Sharif Y, Bhatti HN (2010) Enhanced production of ligninolytic enzymes by ganoderma lucidum IBL-06 using lignocellulosic agricultural wastes. Int J Chem React Eng 8(1). https://doi.org/10.2202/1542-6580.2203

89. Holt GA et al (2012) Fungal mycelium and cotton plant materials in the manufacture of biodegradable molded packaging material: evaluation study of select blends of cotton byproducts. J Biobased Mater Bioenergy 6(4):431–439. https://doi.org/10.1166/jbmb.2012.1241
90. Hemery Y, Rouau X, Lullien-Pellerin V, Barron C, Abecassis J (2007) Dry processes to develop wheat fractions and products with enhanced nutritional quality. J Cereal Sci. The Contribution of Cereals to a Healthy Diet 46(3):327–347. https://doi.org/10.1016/j.jcs.2007.09.008
91. Song L, Ma F, Zeng Y, Zhang X, Yu H (2013) The promoting effects of manganese on biological pretreatment with Irpex lacteus and enzymatic hydrolysis of corn stover. Bioresour Technol. Biorefineries 135:89–92. https://doi.org/10.1016/j.biortech.2012.09.004
92. Akpinar M, Ozturk Urek R (2012) Production of ligninolytic enzymes by solid-state fermentation using Pleurotus eryngii. Prep Biochem Biotechnol 42:582–597. https://doi.org/10.1080/10826068.2012.673528
93. Mishra V, Jana AK, Jana MM, Gupta A (2017) Enhancement in multiple lignolytic enzymes production for optimized lignin degradation and selectivity in fungal pretreatment of sweet sorghum bagasse. Bioresour Technol 236:49–59. https://doi.org/10.1016/j.biortech.2017.03.148
94. Chang AJ, Fan J, Wen X (2012) Screening of fungi capable of highly selective degradation of lignin in rice straw. Int Biodeterior Biodegradation 72:26–30. https://doi.org/10.1016/j.ibiod.2012.04.013
95. Angelova MB, Pashova SB, Spasova BK, Vassilev SV, Slokoska LS (2005) Oxidative stress response of filamentous fungi induced by hydrogen peroxide and paraquat. Mycol Res 109(2):150–158. https://doi.org/10.1017/S0953756204001352
96. Li D, Alic M, Brown JA, Gold MH (1995) Regulation of manganese peroxidase gene transcription by hydrogen peroxide, chemical stress, and molecular oxygen. Appl Environ Microbiol 61(1):341–345. https://doi.org/10.1128/aem.61.1.341-345.1995
97. Asther M, Corrieu G, Drapron R, Odier E (1987) Effect of tween 80 and oleic acid on ligninase production by phanerochaete chrysosporium INA-12. Enzym Microb Technol 9(4):245–249. https://doi.org/10.1016/0141-0229(87)90024-X
98. Sánchez Muñoz S et al (2021) Surfactants in biorefineries: role, challenges & perspectives. Bioresour Technol 345:126477. https://doi.org/10.1016/j.biortech.2021.126477
99. Andersson R, Westerlund E, Tilly AC, Åman P (1993) Natural variations in the chemical composition of white flour. J Cereal Sci 17(2):183–189. https://doi.org/10.1006/jcrs.1993.1018
100. Ballesteros LF, Teixeira JA, Mussatto SI (2014) Chemical, functional, and structural properties of spent coffee grounds and coffee silverskin. Food Bioprocess Technol 7(12):3493–3503. https://doi.org/10.1007/s11947-014-1349-z
101. Barbero-López A, Monzó-Beltrán J, Virjamo V, Akkanen J, Haapala A (2020) Revalorization of coffee silverskin as a potential feedstock for antifungal chemicals in wood preservation. Int Biodeterior Biodegradation 152:105011. https://doi.org/10.1016/j.ibiod.2020.105011
102. Bresciani L, Calani L, Bruni R, Brighenti F, Del Rio D (2014) Phenolic composition, caffeine content and antioxidant capacity of coffee silverskin. Food Res Int. Coffee – Science, Technology and Impacts on Human Health 61:196–201. https://doi.org/10.1016/j.foodres.2013.10.047
103. Borrelli RC, Esposito F, Napolitano A, Ritieni A, Fogliano V (2004) Characterization of a new potential functional ingredient: coffee silverskin. J Agric Food Chem 52(5):1338–1343. https://doi.org/10.1021/jf034974x
104. Foti MC (2007) Antioxidant properties of phenols. J Pharm Pharmacol 59(12):1673–1685. https://doi.org/10.1211/jpp.59.12.0010
105. Li Z, Zhang J, Qin L, Ge Y (2018) Enhancing antioxidant performance of lignin by enzymatic treatment with laccase. ACS Sustain Chem Eng 6(2):2591–2595. https://doi.org/10.1021/acssuschemeng.7b04070

106. Tripathi JP, Yadav JS (1992) Optimisation of solid substrate fermentation of wheat straw into animal feed by Pleurotus ostreatus: a pilot effort. Anim Feed Sci Technol 37(1):59–72. https://doi.org/10.1016/0377-8401(92)90120-U
107. Yadav JS (1987) Influence of nutritional supplementation on solid-substrate fermentation of wheat straw with an alkaliphilic white-rot fungus (Coprinus sp.). Appl Microbiol Biotechnol 26(5):474–478. https://doi.org/10.1007/BF00253535
108. Yadav JS, Tripathi JP (1991) Optimization of cultivation and nutrition conditions and substrate pretreatment for solidsubstrate fermentation of wheat straw by Coriolus versicolor. Folia Microbiol 36(3):294–301. https://doi.org/10.1007/BF02814364
109. Haneef M et al (2017) Advanced materials from fungal mycelium: fabrication and tuning of physical properties. Sci Rep 7(1):41292. https://doi.org/10.1038/srep41292
110. Islam MR, Tudryn G, Bucinell R, Schadler L, Picu RC (2017) Morphology and mechanics of fungal mycelium. Sci Rep 7(1):13070. https://doi.org/10.1038/s41598-017-13295-2
111. Antinori ME, Ceseracciu L, Mancini G, Heredia-Guerrero JA, Athanassiou A (2020) Fine-tuning of physicochemical properties and growth dynamics of mycelium-based materials. ACS Appl Bio Mater 3(2):1044–1051. https://doi.org/10.1021/acsabm.9b01031
112. Islam MR, Tudryn G, Bucinell R, Schadler L, Picu RC (2018) Stochastic continuum model for mycelium-based bio-foam. Mater Des 160:549–556. https://doi.org/10.1016/j.matdes.2018.09.046
113. Green DW, Winandy JE, Kretschmann DE (1999) Mechanical properties of wood. In: Wood handbook: wood as an engineering material, p 113
114. Shen Y-L, Finot M, Needleman A, Suresh S (1994) Effective elastic response of two-phase composites. Acta Metall Mater 42(1):77–97. https://doi.org/10.1016/0956-7151(94)90050-7
115. Curling S, Clausen C, Winandy J (2002) Relationships between mechanical properties, weight loss, and chemical composition of wood during incipient brown-rot decay. For Prod J 52(7):34–39
116. Selig ET, Roner CJ (1987) Effects of particle characteristics on behavior of granular material. In: Transportation research record, 1131
117. Appels FVW et al (2019) Fabrication factors influencing mechanical, moisture- and water-related properties of mycelium-based composites. Mater Des 161:64–71. https://doi.org/10.1016/j.matdes.2018.11.027
118. Gou L, Li S, Yin J, Li T, Liu X (2021) Morphological and physico-mechanical properties of mycelium biocomposites with natural reinforcement particles. Constr Build Mater 304:124656. https://doi.org/10.1016/j.conbuildmat.2021.124656
119. He J, Cheng CM, Su DG, Zhong MF (2014) Study on the mechanical properties of the latex-mycelium composite. Appl Mech Mater 507:415–420. https://doi.org/10.4028/www.scientific.net/AMM.507.415
120. Arvidsson R, Nguyen D, Svanström M (2015) Life cycle assessment of cellulose nanofibrils production by mechanical treatment and two different pretreatment processes. Environ Sci Technol 49(11):6881–6890. https://doi.org/10.1021/acs.est.5b00888
121. Sun W, Tajvidi M, Hunt CG, McIntyre G, Gardner DJ (2019) Fully bio-based hybrid composites made of wood, fungal mycelium and cellulose nanofibrils. Sci Rep 9(1):3766. https://doi.org/10.1038/s41598-019-40442-8
122. Jiang L, Walczyk D, McIntyre G, Bucinell R, Tudryn G (2008) Manufacturing of biocomposite sandwich structures using mycelium-bound cores and preforms. Appl Biochem Biotechnol 146(1):50–59. https://doi.org/10.1016/j.jmapro.2017.04.029
123. Sàez D, Grizmann D, Trautz M, Werner A (2021) Developing sandwich panels with a mid-layer of fungal mycelium composite for a timber panel construction system. In: Proceedings of the 2021 world conference on timber engineering
124. Ziegler AR, Bajwa SG, Holt GA, McIntyre G, Bajwa DS (2016) Evaluation of physico-mechanical properties of mycelium reinforced green biocomposites made from cellulosic fibers. Appl Eng Agric 32(6):931–938. https://doi.org/10.13031/aea.32.11830

125. Elsacker E, Søndergaard A, Van Wylick A, Peeters E, De Laet L (2021) Growing living and multifunctional mycelium composites for large-scale formwork applications using robotic abrasive wire-cutting. Constr Build Mater 283:122732. https://doi.org/10.1016/j.conbuildmat.2021.122732
126. Elsacker E et al (2021) Mechanical characteristics of bacterial cellulose-reinforced mycelium composite materials. Fungal Biol Biotechnol 8(1):18. https://doi.org/10.1186/s40694-021-00125-4
127. López Nava JA, Méndez González J, Ruelas Chacón X, Nájera Luna JA (2016) Assessment of edible fungi and films bio-based material simulating expanded polystyrene. Mater Manuf Process 31(8):1085–1090. https://doi.org/10.1080/10426914.2015.1070420
128. Elsacker E, Vandelook S, Brancart J, Peeters E, Laet LD (2019) Mechanical, physical and chemical characterisation of mycelium-based composites with different types of lignocellulosic substrates. PLoS One 14(7):e0213954. https://doi.org/10.1371/journal.pone.0213954
129. Heisel F et al (2018) Design, cultivation and application of load-bearing mycelium components: the MycoTree at the 2017 Seoul Biennale of architecture and urbanism. Int J Sustain Energy Dev 6(1):296–303. https://doi.org/10.20533/ijsed.2046.3707.2017.0039
130. Travaglini S, Noble J, Ross PG, Dharan CKH (2013) Mycology matrix composites. In: Proceedings of the American Society for composites twenty-eighth technical conference, pp 517–535. https://doi.org/10.1017/CBO9781107415324.004
131. Travaglini S, Dharan CKH, Ross PG (2014) Mycology matrix sandwich composites flexural characterization. In: Proceedings of the American Society for composites 29th technical conference. DEStech Publications, Inc., pp 1941–1955
132. Lelivelt R, Lindner G, Teuffel P, Lamers H (2015) The production process and compressive strength of Mycelium-based materials. In: First International Conference on bio-based building materials, pp 1–6
133. Bruscato C, Malvessi E, Brandalise RN, Camassola M (2019) High performance of macrofungi in the production of mycelium-based biofoams using sawdust—sustainable technology for waste reduction. J Clean Prod 234:225–232. https://doi.org/10.1016/j.jclepro.2019.06.150
134. Sivaprasad S, Byju SK, Prajith C, Shaju J, Rejeesh CR (2021) Development of a novel mycelium bio-composite material to substitute for polystyrene in packaging applications. Mater Today: Proc. https://doi.org/10.1016/j.matpr.2021.04.622
135. Ongpeng J, Inciong E, Siggaoat A, Soliman CA, Sendo VB (2020) Using waste in producing bio-composite mycelium bricks. Appl Sci 10. https://doi.org/10.3390/app10155303
136. Rigobello A, Ayres P (2021) Fragile computation: rethinking information technologies to foster situated ecologies. In: Proceedings of the deep city 2021 conference. (in press)
137. Rigobello A, Gaudillière-Jami N (2021) Designing the gross. In search for social inclusion. In: Design culture(s). Cumulus Conference Proceedings Roma, vol 2, pp 811–827
138. Cerimi K, Akkaya KC, Pohl C, Schmidt B, Neubauer P (2019) Fungi as source for new bio-based materials: a patent review. Fungal Biol Biotechnol 6(1):17. https://doi.org/10.1186/s40694-019-0080-y

Development of Building Insulation Material from Fungal Mycelium

21

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Abstract

In the light of environmental impact caused by conventional insulation materials, there is an increased interest in the scientific community to develop new insulating materials from renewable sources. Mycelium-based biocomposites have been found to be promising for sustainable packaging, acoustic and thermal insulation, fashion and architecture. Mycelium-based biocomposite insulation materials contribute to reduce the environmental footprint of buildings. These composites have a significant advantage in terms of thermal conductivity and fire safety over traditional synthetic insulation materials. This chapter starts with a review of the scientific literature on the mycelium-based biocomposites, which have been used for various applications. In addition, the main information as regards to manufacturing an eco-friendly insulation material using *Miscanthus giganteus* and fungal mycelium is discussed. Two fungi (*Ganoderma resinaceum* and *Pleurotus ostreatus*) were used to produce biocomposites. In order to find the most suitable mixture as building insulation material as regards to density and thermal conductivity, mixing different proportions of *Miscanthus* and mycelium were used. The results are promising to make a sustainable insulation material based on fungal mycelium and *Miscanthus* fibres.

Keywords

Mycelium · *Miscanthus* · Biocomposites · Thermal insulation · Thermal conductivity · Fire safety

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21.1 Introduction

The building industry is moving towards new approaches not only to protect the environment by reducing the overall footprint of buildings but also to improve the thermal performance of the building envelope by reducing the embodied energy of the materials. Thermal insulation materials are generally used in the construction industry to ensure adequate performance and to allow significant energy savings. Most of these materials are made from synthetic compounds, such as expanded polystyrene and polyurethane, which are far less sustainable than bio-based materials. In light of the environmental impact caused by manufacturing synthetic materials, intensive research efforts are being made to develop sustainable new eco-friendly biomaterials with a wide variety of properties to replace the traditional building insulation materials that exhibit high-embodied energy [1].

The use of agricultural crop residues as raw materials for innovative bio-based building insulation products had been suggested as sustainable alternative for traditional synthetic materials [2]. Insulation materials from natural materials and agricultural crop wastes, such as cork, hemp, flax, rice husk and kenaf, have been shown to be competitive hygrothermal performance and lower cost as compared to commercially available synthetic insulation materials [3, 4]. One of the important features of this kind of materials is their biodegradability.

Several researchers have evaluated the thermal performance of insulation materials based on crop by-products. Xu et al. [5] reported the value of thermal conductivity of 0.04–0.065 W m^{-1} K^{-1} in kenaf binderless particleboards. Pinto et al. [6] showed that corncob panels have the potential to be used as a thermal insulation material, which has a thermal conductivity value of 0.139 W m^{-1} K^{-1}. Gaujena et al. [7] also studied insulation panels made from hemp fibres, and reported thermal conductivity ranging between 0.0544 and 0.0594 W m^{-1} K^{-1}.

Recently, there is a considerable attention on the *Miscanthus* among agricultural by-products due to its porous structure and favourable properties [8]. The thermal conductivity of *Miscanthus* fibres is 0.04 W m^{-1} K^{-1}, which is equivalent to the thermal conductivity of traditional insulation materials available in the market [9]. In France, low-cost insulation particle board panels were manufactured using *Miscanthus* and Sunflower stalks, demonstrating their potential due to their low density and good thermal conductivity [10]. El Hage et al. [11] developed a novel thermal insulating biocomposite using *Miscanthus* and recycled textile fibres bonded with chitosan that has a thermal conductivity value between 0.069 and 0.09 W m^{-1} K^{-1}.

Being a cheap, effective and environment-friendly product, the use of mycelium has been recently found to produce biocomposites as new biodegradable materials [12, 13]. Mycelium-based composites yield low density with higher strength and lower embodied energy compared to synthetics composites [13]. Mycelium-based composite materials are made by growing fungal mycelium on plant-based substrates such as hemp, sawdust, straw and cotton; these are very promising biodegradable and non-toxic materials to replace traditional synthetic materials, due to their low environmental impact, energy consumption, density and cost, as well as their good insulating, acoustic and fire performance [12, 14–17].

This chapter starts with a review of a variety of the fungi and feeding substrates used in manufacturing mycelium-based composites as well as their potential applications. The differences in growth rates of mycelia from two fungal strains (*Ganoderma resinaceum* and *Pleurotus ostreatus*) are also discussed. Discussion on the novel mycelium-based composites using *Miscanthus* fibres that could replace conventional insulation materials, such as expanded polystyrene and extruded polystyrene, is also presented.

21.2 Mycelium and Its Products

21.2.1 Mycelium

Fungi play an important role as nutrient recycling in nature and it is estimated that there may exist 2.2–3.8 million fungal species around the world [18]. Mycelium is the vegetative lower part of fungi in the substratum. Fungi are considered as one of the largest groups of living organisms on earth [19] that grow on a nutrient substrate (Fig. 21.1a).

Mycelium has a unique ability to form composite materials quickly and has many biological functions such as nutrition, growth and the maintaining of its host [20]. As mycelium grows on materials, such as biological and agricultural wastes, it penetrates its substratum containing nutrients by physical pressure and enzymic secretion to degrade the host materials while simultaneously binding them together [21]. Within right conditions, the fruiting bodies of basidiomycete fungi, generally known as mushrooms, can sprout from the mycelial network. Figure 21.1b shows how mycelium has a porous structure composed of a mass of branched network of tiny filaments called hyphae [22]. These hyphae which have diameters in the range of 1–30 μm and lengths ranging from a few microns to several meters [23] form a

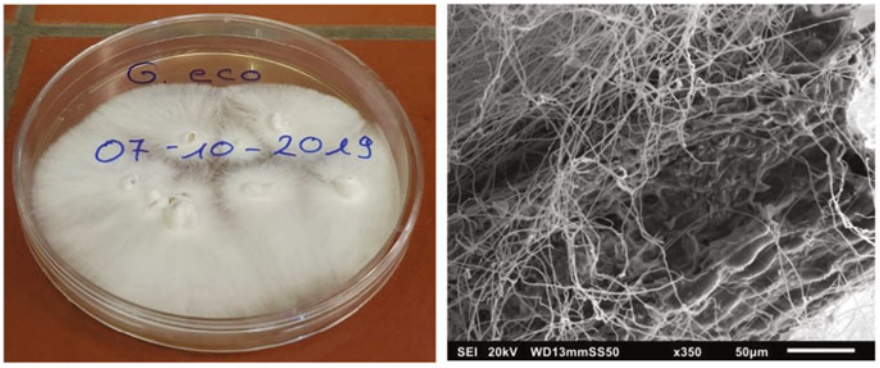


Fig. 21.1 (**a**) Mycelium growth and (**b**) mycelium hyphae

three-dimensional network of hyphae by growing at their tips and by interconnecting randomly.

Jiang et al. [24] explained that mycelium acts like a natural glue that spreads in every direction and binds firmly to natural materials with no added energy. Its growth rate depends on the type of fungus, temperature, humidity and nutrient medium [25]. High temperatures are important for the initial growth rate, but they do not grow at extreme temperatures [26]. For example, the fungal group of *Basidiomycota* does not grow above 40 °C [27]. In general, most species need temperature around 25 °C with high humidity [12].

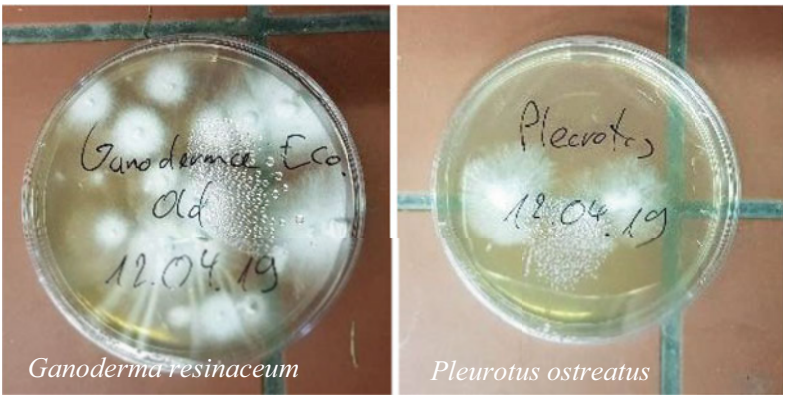
According to Jones et al. [14], substrates are often enriched with carbon, nitrogen, oxygen, sulphur, phosphorous, potassium and magnesium in millimolar concentrations and hydrogen, calcium, copper, iron, manganese, zinc, nickel and molybdenum in micromolar concentrations, which provide the nutrition necessary for the mycelium to grow. For ideal mycelial growth, substrates high in sugars such as glucose, cellulose, lignin and starch are widely used [28]. The type and size of substrate particles have a significant effect on the properties of the mycelium. Various agricultural materials, such as rice hulls [15, 29], wheat straw [30, 31], hemp [27, 30, 32, 33], sawdust [31, 34, 35] and corn stover, [36, 37] have been used as substrates to provide nutrients for the growth and expansion of mycelium in order to produce mycelium-based composites.

In a preliminary experiment by the authors, two fungal species of *Ganoderma resinaceum* and *Pleurotus ostreatus* were chosen to observe the difference in the growth of mycelium. *Ganoderma* belongs to the family of the macro-mushroom Ganodermataceae, which own more than 300 different species. These species can be found all over the world in different characteristics such as the shape and colour of the mushroom. The mycelium from the *Ganoderma* family develops better than other fungal species. *Ganoderma resinaceum* is a very active fungus in the medical sector [38]. Its mycelial growth rate increases with increase in temperature. However, the optimal growth rate is at 30 °C, whereas when the temperature exceeds 35 °C, the mycelium growth is reduced significantly [38].

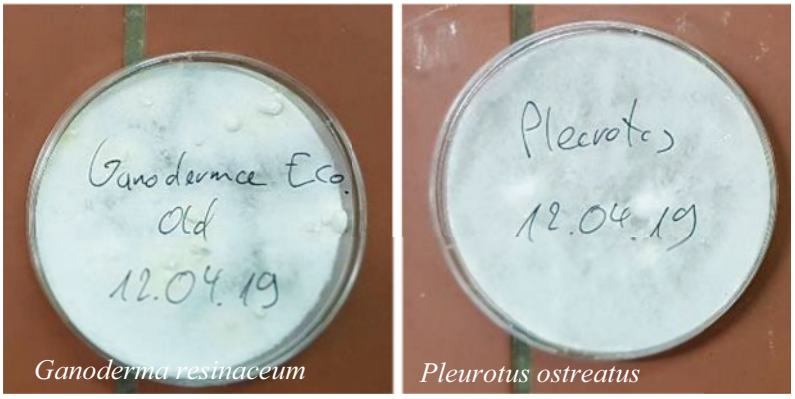
The other mushroom type is the *Pleurotus*, which belong to the family Pleurotaceae. *Pleurotus ostreatus* is also known as oyster mushroom, which includes one of the most widely edible mushrooms. They are distributed all over the world and are mostly found in forests where they are responsible for the decomposition of wood and plant residues. High humidity (relative humidity 98%) and warm room temperature (25–30 °C) provide an excellent environment for the growth of its mycelium [39].

Petri dishes inoculated with two fungal species were subsequently placed in an incubator at room temperature (around 20–22 °C) to allow the fungi to grow. The development of two fungi was analysed at 3, 5 and 11 days as shown in Fig. 21.2. It can be seen that the mycelium grew after 3 days in the Petri dishes. It was observed that *Pleurotus ostreatus* develops in a more scattered way, while *Ganoderma resinaceum* develops in a wired way but much denser in such a way that it is difficult to distinguish the individual hyphae (Fig. 21.3). It was also observed that *Pleurotus ostreatus* is more likely to develop in-depth than *Ganoderma resinaceum* (Fig. 21.4).

(a)



(b)



(c)

Fig. 21.2 Observations of the growth of mycelium at (**a**) 3 days (**b**) 5 days and (**c**) in 11 days

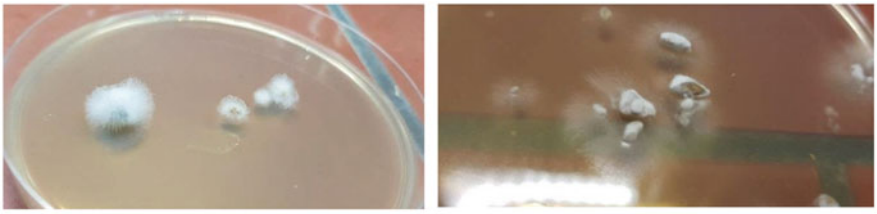


Fig. 21.3 A closer view of the mycelium growth of two strains (**a**) *Ganoderma resinaceum* and (**b**) *Pleurotus ostreatus* at 3 days

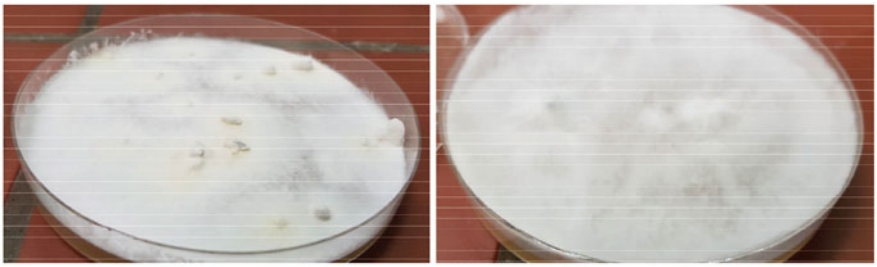


Fig. 21.4 A closer view of the mycelium growth of two strains (**a**) *Ganoderma resinaceum* and (**b**) *Pleurotus ostreatus* after 11 days

21.2.2 Mycelial Composites

Due to the high growth rates and sustainability of mycelium, several researchers explored to produce mycelium-based composites and evaluated their applicability in various industries, such as packaging [12, 16, 40], insulating panels [33, 41], papers [42, 43] and wearables [44, 45]. Table 21.1 summarizes previous reports on mycelium-based composites.

Cerimi et al. [62] reported that so far 27 fungal species have been used for the development of mycelium-based materials. As reported in Table 21.1, *Ganoderma lucidum* and *Pleurotus ostreatus* species have been mostly used for the development of mycelium-based composites. Arifin and Yusuf [15] found that a composite made from mycelium can be used to replace polystyrene foam due to its biodegradability, renewability and lack of toxic components. However, the quality and properties of mycelium-based materials can be controlled by the characteristics of the substrate and the fungal species as well as the environmental conditions during growth and processing method [55]. Holt et al. [16] found that a mycelium composite based on *Ganoderma* species and cotton plant materials can be used as a substitute for polystyrene in the packaging industries.

Table 21.1 Previous reports on mycelium-based composites in the literature

Reference	Fungal species	Substrates	Target applications
Arfin and Yusuf [15]	Not specified	Rice husk and wheat grain	Packaging
Holt et al. [16]	*Ganoderma* sp.	Cotton carpel, cottonseed hull, starch and gypsum	Packaging
Pelletier et al. [33]	*Basidiomycetes*	Switchgrass, rice straw, sorghum fibre, flax shive, kenaf and hemp	Acoustic insulation
Travaglini et al. [46]	*Ganoderma lucidum*	Red oak wood chips and a nutrient solution (not specified)	Packaging, insulation and sandwich panels
He et al. [47]	*Pleurotus ostreatus*	Cottonseed hulls, carboxylated styrene–butadiene rubber latex, Silane coupling agent	Not specified
Lelivelt et al. [27]	*Trametes versicolor* and *Pleurotus ostreatus*	Wood chips, hemp curd, loose hemp fibre and non-woven, mats of hemp fibre	Replace fossil-based plastics
Mayoral Gonzalez and Gonzalez Diez [48]	*Lentinula edodes*, *Pleurotus ostreatus* and *Ganoderma lucidum*	Wood shavings, straw, corn stalk and rice husks	Thermal insulating structures
Ziegler et al. [40]	Not specified	Cotton ginning waste and hemp pith	Packaging
Jiang et al. [13]	Not specified	Natural materials (not specified)	Sandwich structures
López Nava et al. [49]	*Pleurotus ostreatus*	Crop residues (*Triticum* sp.) and edible films (carrageenan, chitosan and xanthan gum)	Food packaging
Jiang et al. [24]	Not specified	Kenaf and hemp	Perform shell
Yang et al. [41]	*Basidiomycete* saprobes	Alaska birch, millet grain, wheat bran, natural fibre and calcium sulphate	Backfill material for geoengineering applications and insulation for building and infrastructure
Pelletier et al. [50]	*Basidiomycetes*	Cotton burs, switchgrass, rice straw, sorghum stalks, kenaf and corn stalks	Acoustic insulation
Haneef et al. [17]	*Pleurotus ostreatus* and *Ganoderma lucidum*	Pure cellulose and cellulose–potato dextrose	Mycelium films

(continued)

Table 21.1 (continued)

Reference	Fungal species	Substrates	Target applications
Dahmen [51]	Not specified	Sawdust or agricultural waste and nutrients (not specified)	Furniture
Attias et al. [52]	*Pleurotus ostreatus*, *Pleurotus salmoneostramines*, *Pleurotus pulmonarius* and *Aegerita agrocibe*	Wood chips of eucalyptus, oak, pine, apple and vine	Design and architecture
Campbell et al. [53]	*Pleurotus ostreatus*	Seeds (not specified) mixed with hydrogel	Architectural assembly units
Tudryn et al. [36]	*Basidiomycetes*	Corn stover particles and additives (calcium and carbohydrate)	Composite biopolymer
Jones et al. [54]	*Trametes versicolor*	Rice hulls, glass fines and wheat grains	Insulation and construction materials
Appels et al. [55]	*Pleurotus ostreatus* and *Trametes multicolor*	Rapeseed straw, beech sawdust and non-woven cotton fibres	Product design
Elsacker et al. [30]	*Trametes versicolor*	Hemp, flax, flax waste, softwood and straw	Building materials
Sun et al. [56]	Not specified	A mixture of spruce, pine and for particle–board particles	Packaging and furniture
Matos et al. [57]	*Lentinula edodes*	Coconut powder-based supplemented with wheat bran	Packaging
Attias et al. [58]	*Coriolus* sp., *Trametes* sp. And *Ganoderma* sp.	Woodchips (apple and vine) mixed flour and wheat straw	Design and architecture
Bruscato et al. [34]	*Pycnoporus sanguineus*, *Pleurotus albidus* and *Lentinus velutinus*	Wood sawdust (*Pinus*), wheat bran, agar and calcium carbonate	Expanded polystyrene alternative
Silverman et al. [44]	*Ganoderma lucidum*, *Pleurotus ostreatus*, *Pleurotus eryngii* and *Pleurotus citrinopileatus*	Fabric, sawdust spawn, flour, feathers and psyllium husk	Footwear
Zimele et al. [32]	*Trametes versicolor*	Hardwood chips and hemp shives	Building materials
Ridzqo et al. [59]	*Ganoderma lucidum*	Bamboo fibres	Insulation and partition board
Soh et al. [60]	*Ganoderma lucidum*	Bamboo fibres and chitosan	Structures
Modanloo et al. [35]	*Pleurotus ostreatus*	Shredded paper, wheat bran and guar gum	Architecture
Ding et al. [61]	*Pleurotus ostreatus*	Poplar sawdust and cottonseed hull	Packaging

Pelletier et al. [33] investigated novel eco-friendly mycelium acoustic insulation panels by growing the basidiomycete fungus on various agricultural by-products, such as switchgrass, rice straw, sorghum fibre, flax shive, hemp and kenaf, as an alternative to standard petroleum-based insulation boards. The worst-performing samples have an acoustic absorption rate of over 70–75% at 1000 Hz. It was also concluded that these panels can be applied as sound insulation of mezzanine structures and as an acoustically reflective material. Pelletier et al. [50] carried out a follow-up study on the previous acoustic insulation panel to create a high-density compressed board.

He et al. [47] assessed the compressive strength of mycelial composite produced using *Pleurotus ostreatus* and cottonseed hulls and found that the compressive strength could be increased with the addition of styrene–butadiene rubber. Travaglini et al. [46] also assessed the compressive modulus and strength of *Ganoderma lucidum* mycelial composites grown on a red oak wood sawdust substrate. Lelivelt et al. [27] evaluated the structural performance of biocomposites made from *Trametes versicolor* and *Pleurotus ostreatus* strains grown on different natural substrates. It was found that the combination of hemp mats and the strain *Trametes versicolor* allowed the densest growth of mycelium with a high strength and stiffness. Ziegler et al. [40] conducted a similar research to study the physical and mechanical properties of mycelium-based biocomposite made using cotton gin and hemp pith. López Nava et al. [49] developed a biocomposite using *Pleurotus ostreatus* using crop residues and edible films, such as carrageenan, chitosan and xanthan gum, with similar properties to the expanded polystyrene to be used for food packaging. Yang et al. [41] showed that a new bio-form developed using basidiomycete saprotrophic fungus containing better physical, thermal and mechanical characteristics than the conventional expanded polystyrene foam except density. Haneef et al. [17] used *Ganoderma lucidum* and *Pleurotus ostreatus* species grown on pure cellulose and cellulose–potato dextrose substrates to develop mycelium composites. It was found that the *Pleurotus ostreatus* mycelium on cellulose is stiffer than the *Ganoderma lucidum* mycelium, while the addition of dextrose to the cellulose-based substrate further enhanced the elasticity of materials manufactured by both fungal species.

Attias et al. [52] used four fungal species, including *Pleurotus ostreatus*, *Pleurotus salmoneostramines*, *Pleurotus pulmonarius* and *Aegerita agrocibe* fungi, grown on varied agricultural wastes to evaluate the most suitable combination for future applications in design and architecture. The research showed that the *Pleurotus ostreatus* mycelium grown on vine or apple substrates was the most promising for producing mycelium-based composites. Matos et al. [57] explored methods of producing a biodegradable composite for packaging using edible mushroom *Lentinula edodes* cultivated in coconut powder and wheat bran. Bruscato et al. [34] produced biofoams, which has compressive strength 60% greater than that of expanded polystyrene, using three types of white-rot fungi *Pycnoporus sanguineus*, *Pleurotus albidus* and *Lentinus velutinus* grown on a substrate made of *Pinus* sawdust and wheat bran.

21.2.3 Mycelial Composites for Insulation Applications

There is limited scholarly research on the use of mycelium-based biocomposites in thermal insulation applications for building envelopes. A few of these products exist in the marketplace.

Cadena and Bula [63] used rice husks and yucca starch to manufacture biocomposite insulation boards, which have a thermal conductivity of 0.065 W m^{-1} K^{-1} and a density of 195 kg/m^3. Jones et al. [29] developed *Trametes versicolor* mycelium composite grown on rice hulls and assessed its thermal degradation and fire reaction properties with commercially available extruded polystyrene foam. It was found that the high silica content of rice hulls contributes to the better fire reaction properties of the biocomposite. Yang et al. [41] produced a mycelium-based biocomposite that has a thermal conductivity between 0.05 and 0.07 W m^{-1} K^{-1} and a density between 160 and 280 kg/m^3. The larger thermal conductivity can be attributed to the higher density of the material [30, 64, 65]. Xing et al. [66] used *Oxypous latermarginatus*, *Megasporoporia minor* and *Ganoderma resinaceum* species grown on wheat straw substrates in order to develop novel bio-based insulation materials. The thermal conductivity of these biocomposites varied between 0.078 and 0.081 W m^{-1} K^{-1}.

Elsacker et al. [30] examined the thermal conductivity of mycelium composites with different types of fibres such as hemp, flax and straw combined with fungal mycelium of *Trametes versicolor*. These composites were reported to have thermal conductivities of 0.0404–0.0578 W m^{-1} K^{-1}. It was also found that these composites have an overall good insulation behaviour in all aspects compared to conventional insulation materials including rock wool, glass wool, kenaf and expanded polystyrene.

Wimmers et al. [67] studied the novel use of mycelium of several wood-decaying *Basidiomycetes* grown on sawdust and wood shavings in thermal insulation applications for buildings. Wimmers et al. [67] obtained thermal conductivity values between 0.051 and 0.055 W m^{-1} K^{-1} for mycelium-based composites, which makes it a promising material for thermal insulation. Holt et al. [16] found thermal conductivity values between 0.10 and 0.18 W m^{-1} K^{-1} when *Ganoderma* species and cotton plant materials were used, while Schritt et al. [68] revealed thermal conductivities between 0.06 and 0.07 W m^{-1} K^{-1} when *Ganoderma lucidum* and beech sawdust were used.

21.3 Past Experience in Producing Mycelium–*Miscanthus* Insulation

The researchers at the University of Luxembourg have been conducting investigations on the development of novel building materials incorporating locally available crop by-products for the reduction of CO_2 emissions, energy consumption and natural resource utilization in the building industry.

Fig. 21.5 Grown *Miscanthus giganteus* [12]

To this end, Pereira Dias and Waldmann [69] focused on producing lightweight concrete by substituting the traditional aggregates with *Miscanthus* due to its wide availability in Luxembourg. *Miscanthus giganteus* is a tall rhizomatous grass native to Eastern Asia (Fig. 21.5). It has about 40% of cellulose and 15% of lignin [70] and has high rigidity with low density [71]. Pereira Dias and Waldmann [69] found that the best theoretical mixture considering the compressive strength of the *Miscanthus* concrete would constitute 150 kg/m^3 of *Miscanthus* and 592 kg/m^3 of cement with 0.8 of water/cement ratio. Then, *Miscanthus* concrete mixture was also used to manufacture masonry blocks [72]. Furthermore, Pereira Dias et al. [73] developed a machine learning tool to optimize the mix design of *Miscanthus* lightweight concrete according to the needs in compressive strength.

Since the innovation of bio-based materials made from renewable resources would make the economy sustainable, the authors investigated the possibility of introducing a self-growing insulation material using the fungal mycelium with *Miscanthus* fibres that could replace conventional insulation materials. Considering the working environment, two fungal strains, *Ganoderma resinaceum* and *Pleurotus ostreatus*, were chosen to produce the test samples in this study (Fig. 21.6). The details of the procedure are presented by Pereira Dias et al. [74]. Here, only the main aspects are reported.

21.3.1 Preparation of Insulation Panels

The process to prepare mycelium-based biocomposite by the authors at the Laboratory of Solid Structures of the University of Luxembourg consisted of six different steps, which are schematically illustrated in Fig. 21.7.

Fig. 21.6 Fruiting body of the (**a**) *Ganoderma resinaceum* [38] and (**b**) *Pleurotus ostreatus*

21.3.1.1 Preparation of Substrate

The manufacturing of the material began with preparing the substrate. In order to obtain the optimal mix proportions of each constituent to produce a lightweight biocomposite, several quantity ratios of the different raw materials were used. For each mixture, 150 g of dried and chopped *Miscanthus* fibres with an average density of 120 kg/m^3 were used to prepare the substrate. To enrich the substrate and to stimulate the growth of mycelium, it was decided to add an additive (potato starch or used coffee grounds) mixed with 1.5 L of water in the mixture. The mixing procedure was carried out in a sterilized condition and the mixture was put into boiling water and pasteurized for 30 min to reduce the number of bacteria in the substrate. After the pasteurization, it is important to filter out the excess water and cool down the wet substrate before the inoculation stage, which involves the addition of active mycelium to the substrate.

21.3.1.2 Mixing Mycelium with the Substrate

The mycelium was kept at 4 °C and was removed from the refrigerator just before use, in order to prevent the destruction of the mycelium due to the heat. Once the substrate reached the desired temperature (about 30–35 °C), the fungus mycelium was added and mixed until a homogeneous mixture was obtained.

21.3.1.3 Preparation of Test Samples

Next, the mixture was transferred into moulds. In order to study the hygrothermal properties of the biocomposite, the prism moulds with the dimensions of 40 mm × 40 mm × 160 mm and the rectangular wooden forms with dimensions of 500 mm × 500 mm × 70 mm were used to manufacture the test samples. Before the mixture was put into the moulds, they were disinfected and covered with sterilized plastic wraps to avoid direct contact between the mixture and mould and to maintain a certain humidity level to help the growth of the fungi in the incubation stage, which is the stage that allows colonization of the substrate by mycelium.

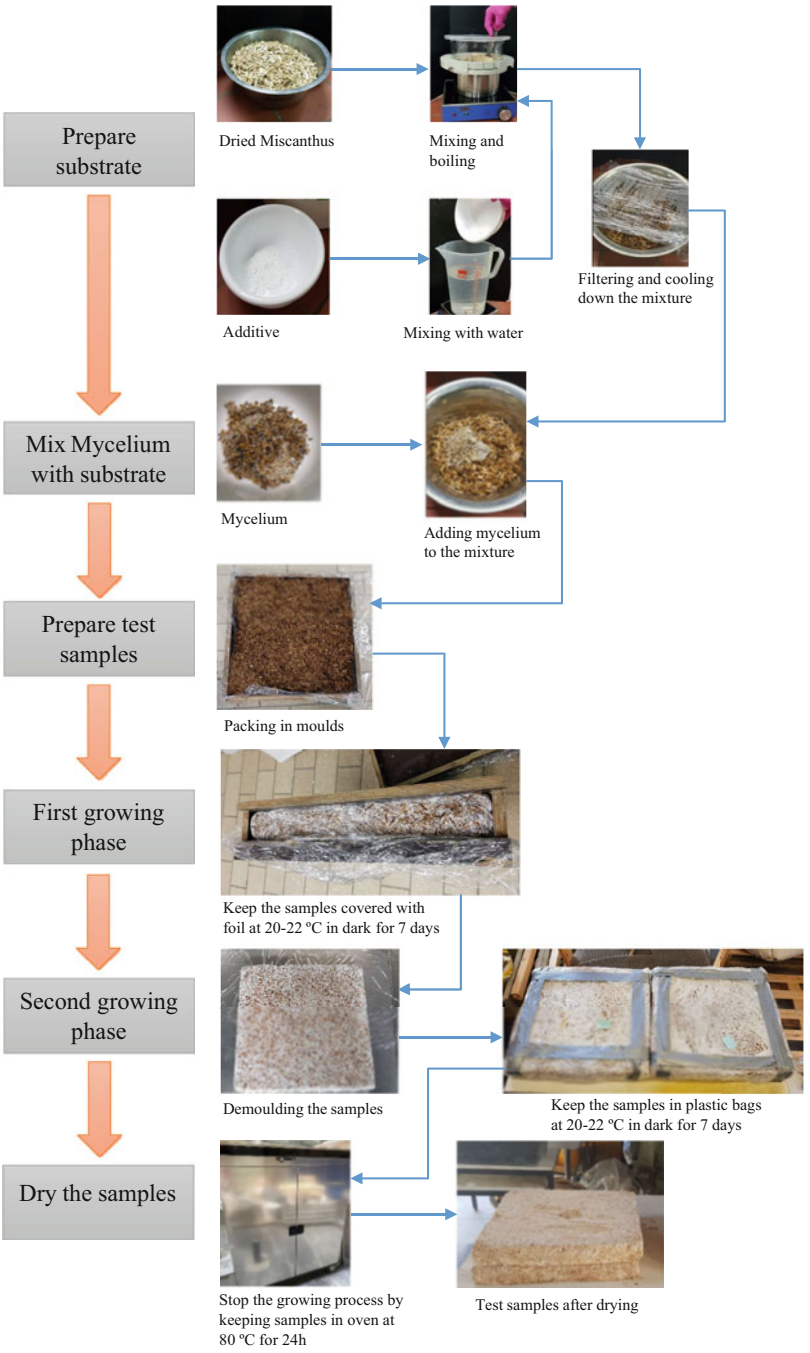


Fig. 21.7 Manufacturing procedure of test samples

21.3.1.4 First Growth Phase

The growth of the fungus does not need extra energy input, however, requires special conditions in terms of temperature and humidity. The mycelium can be grown in a mould to build different shapes and they grow quickly into a solid material. The growing phase of the fungus should take place under sterile conditions in order to counteract external organisms from harming the growth of the mycelium, under high humidity, darkness and room temperature. The incubation period for the test samples was considered into two growth phases.

The first growth phase was limited to 7 days at room temperature (around 20–22 °C). During this period, the samples were kept in a dark closed cabinet.

21.3.1.5 Second Growth Phase

After 7 days, the samples were taken out from the cabinet, demoulded and measured the dimensions and weights of the test samples. During the second growth phase, the samples were then put separately in sterilized bags. The bags were closed by leaving considerable space for better inner oxygenation and a humidity level to stimulate mycelium growth towards a denser network and obtain a better homogeneity on the surfaces. The samples were incubated in the same cabinet at room temperature only for 10 days to avoid the growth of fruiting bodies.

21.3.1.6 Dry the Samples

At the end of the second growth phase, once reaching the desired density and shape, the samples were measured and weighed again. Then, the material should be dehydrated at 80 °C, so that the fungus stops to grow further. The samples were measured and weighted repeatedly every hour. The drying process was stopped when the samples reached at least 35% of the initial weight, which could take up to 24 hours.

21.3.2 Density

To get a better view of the weight and volume changes of the samples after each phase, the samples were compared to their densities, as shown in Fig. 21.8. The values shown are the average densities of the three prism samples made from each mixture. The first letter in each mixture represents the fungal specie used (G – *Ganoderma resinaceum* and P – *Pleurotus ostreatus* mushroom). The second letter, i.e. M, represents the *Miscanthus* fibres, while the third letter describes the additives used for the preparation of the substrate (S – potato starch and C – used coffee grounds). The number associated with each letter defines the ratio of corresponding material to the amount of *Miscanthus*. As described above, the mixing procedure was carried out under sterilized condition while preparing the samples. However, the last four samples were prepared under non-sterilized condition. Thus, in the mixtures notation, NS represents the non-sterilized condition.

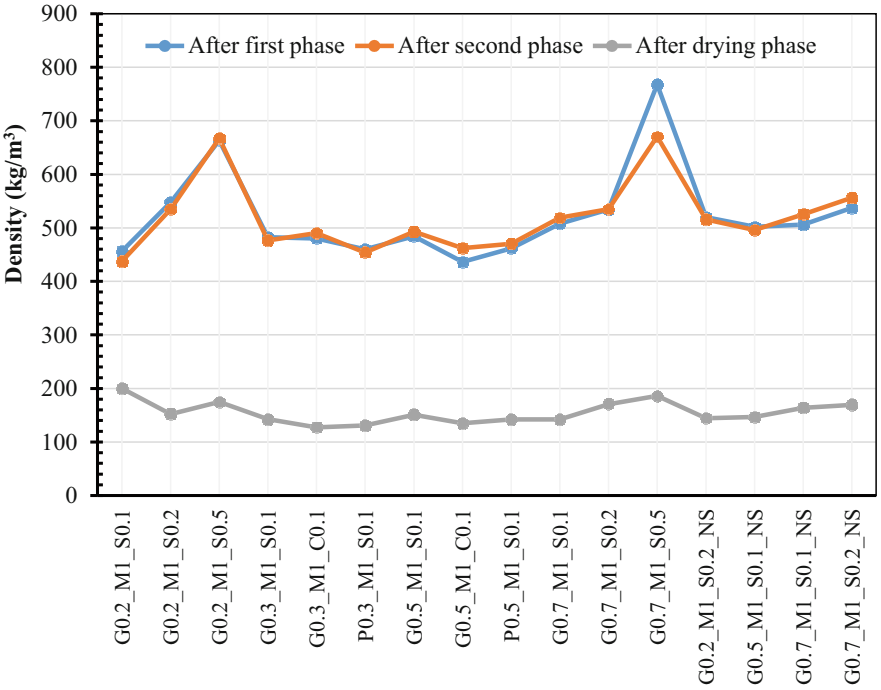


Fig. 21.8 Comparison of average densities of the mixtures after each phase

The density of all composite samples ranged from 127.07 to 200.04 kg/m^3, with the highest density for the mixture of G0.2_M1_S0.1 and the lowest density for the mixture of G0.3_M1_C0.1. The average density obtained from samples made with potato starch is almost similar to that obtained with the coffee grounds.

21.3.3 Visual Observations

Test samples were visually analysed to observe the difference in the growth of mycelium on them. Based on visual analysis, it was seen that all the samples had a stable shape and enough growth of mycelium to hold the body together with a varied amount on the surfaces at the end of the incubation stage (Fig. 21.9).

Moreover, there were no visible differences between the test samples made with potato starch and coffee grounds (i.e. G0.3_M1_S0.1 and G0.3_M1_C0.1, and G0.5_M1_S0.1 and G0.5_M1_C0.1), apart from the fact that during the growth of mycelium, a strong smell of coffee emanates from the samples made with coffee grounds.

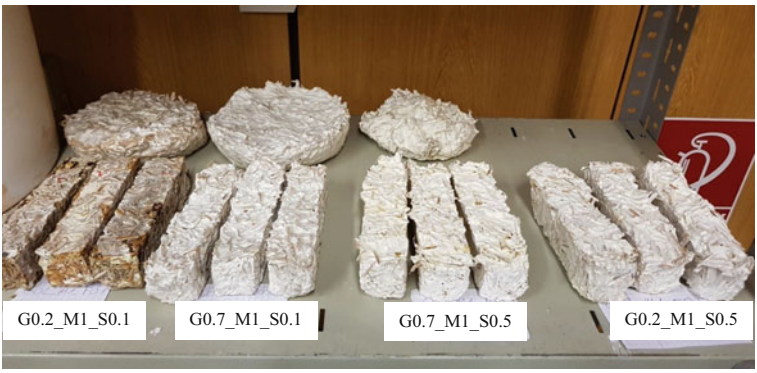


Fig. 21.9 Dried test samples

Fig. 21.10 Damaged dried test samples made with *Pleurotus ostreatus*

The test samples made with *Pleurotus ostreatus* (i.e. P0.3_M1_S0.1 and P0.5_M1_S0.1) at the end of the first and second growth phases confirm the observations made with the Petri dishes, which has been described in Sect. 2.1. These samples had a much more airy and wiry growth of mycelium than the samples made with *Ganoderma resinaceum*, making the final structure much less resistant. The test samples made with *Pleurotus ostreatus* were not chosen to study its because the test samples were falling apart while handling (Fig. 21.10).

21.3.4 Microstructural Characterization

The microstructural formation of the mycelium in the composites was evaluated by performing scanning electron microscopy (SEM) analysis using a JSM-6010LA scanning electron microscope.

SEM analysis was carried out only on selected samples. First, the cell wall structure of the raw samples of *Miscanthus* was observed. Figure 21.11 shows the fibrous network of *Miscanthus*. It indicates that the microstructure of *Miscanthus* is not isotropic and it is formed by numerous hollow tubes, which are all oriented in the same direction.

Several SEM images showed the differences in microstructure between the mycelium and *Miscanthus*. Figure 21.12 shows the SEM images of the formation of mycelium around the *Miscanthus* fibres that were analysed on the test samples of G0.7_M1_S0.5. It can be seen that there was successful mycelium growth within the composite. The white mycelium hyphae could be clearly observable due to their distinct interconnected microstructure when bonded with *Miscanthus* fibres. These hyphae do not create a path through the *Miscanthus* but use what is already available to create a good cohesion between these two components. It can be seen that the mycelium grew within the composite and bonded with the *Miscanthus* fibres.

Figure 21.13 shows the mycelium matrix from sample G0.3_M1_C0.1, while Fig. 21.14 shows the mycelium matrix from sample P0.3_M1_S0.1. Both *Ganoderma* and *Pleurotus* mycelia were interwoven with the larger *Miscanthus* pieces within the composite on feeding different elements. It means that both substrates (i.e. *Miscanthus* used with coffee grounds and *Miscanthus* with potato starch) provided adequate nutrition to allow the mycelium to grow. Based on SEM

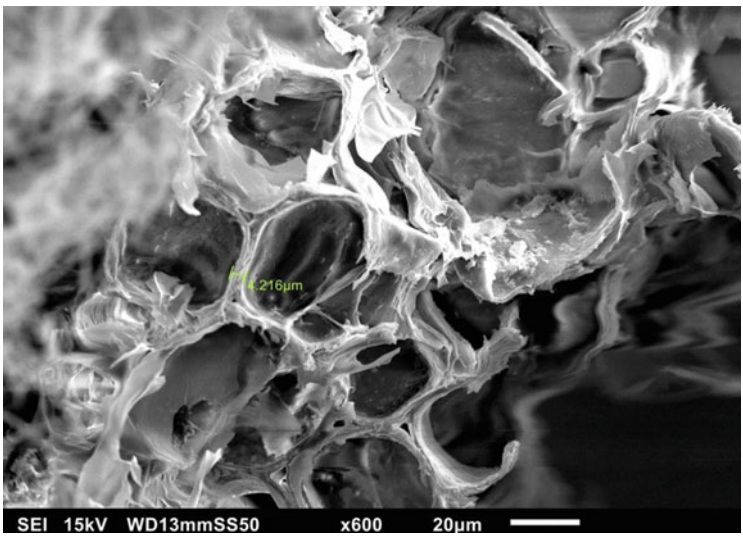


Fig. 21.11 SEM image of *Miscanthus* fibre structure

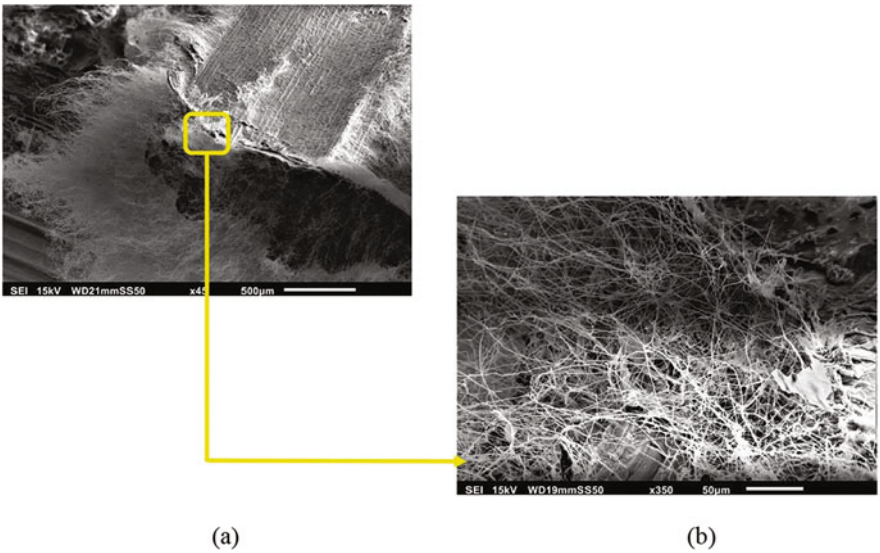


Fig. 21.12 SEM image of sample G0.7_M1_S0.5: (**a**) mycelium bound to *Miscanthus* and (**b**) closer view of the mycelium growth within the composite

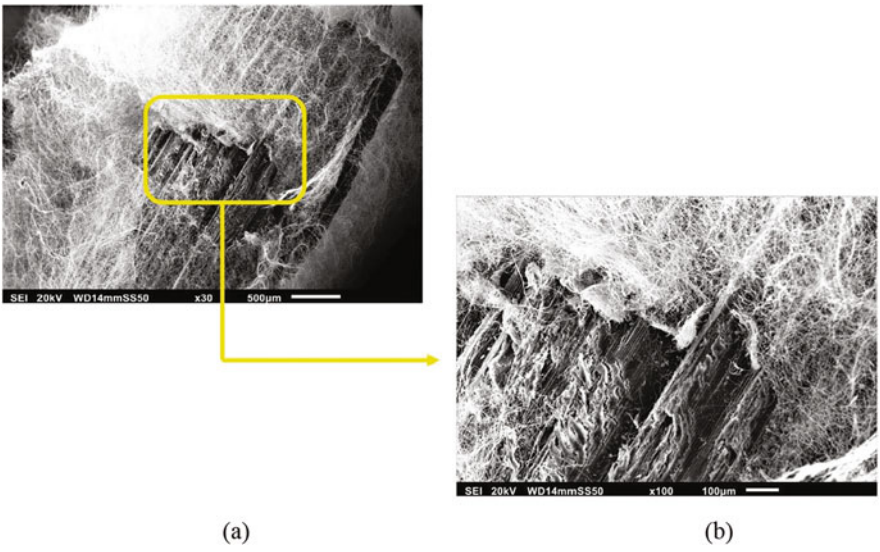


Fig. 21.13 SEM image of sample G0.3_M1_C0.1: (**a**) mycelium bound to *Miscanthus* and (**b**) closer view of the mycelium growth within the composite

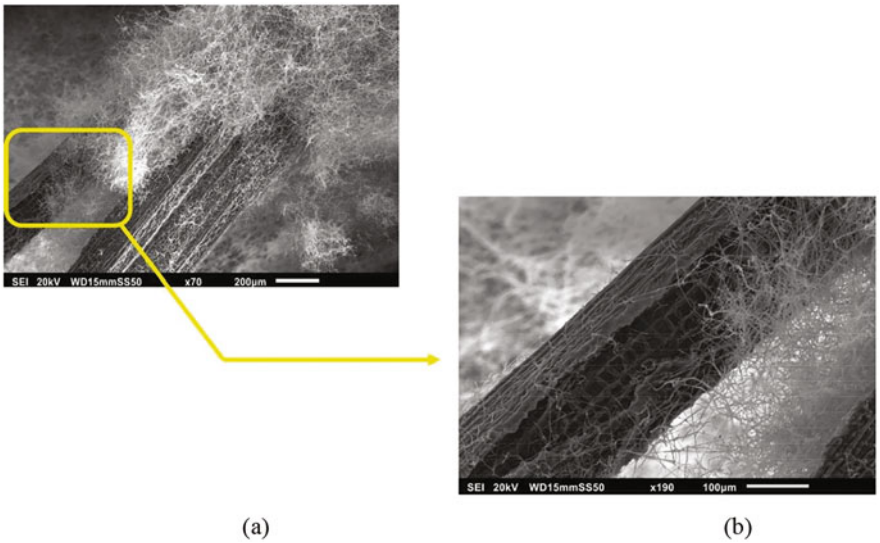


Fig. 21.14 SEM image of sample P0.3_M1_S0.1: (**a**) mycelium bonded with *Miscanthus* and (**b**) closer view of the mycelium growth within the composite

analysis, the porous microstructural formation of the composite is expected to affect the mechanical and thermal properties of the composite.

21.3.5 Thermal Conductivity

In order to study the insulation capacity of the mycelium-based composite, G0.3_M1_S0.1 mix was chosen to manufacture mycelium-based composite plates with the dimensions of 500 mm × 500 mm × 70 mm. The thermal conductivity tests were carried out using Taurus TLP800/900 machine on the composite plate according to ISO 8302/EN 1946-3. For the thermal conductivity tests, composite plates without a render and composite plates with two layers of render according to ETAG-004 [75] were used (Fig. 21.15). More details on the rendering of the plates and the testing procedure can be found in Pereira Dias et al. [74].

The thermal conductivity of the biomaterial was calculated as W m^{-1} K^{-1} (i.e. λ-value) in a permanent state at the temperature difference of 10 K at the top and bottom faces. The variation of thermal conductivity with temperature was analysed for three different mean temperatures at 10, 20 and 30 °C (Fig. 21.16). The thermal conductivity values obtained for two plates without rendering, a single plate without rendering and two plates with rendering were 0.0882, 0.104 and 0.121 W m^{-1} K^{-1}, respectively.

The average density of the samples was 122 kg/m^3. The thermal conductivity value obtained for the new biomaterial falls within the range of the conventional insulation materials, as can be seen in Fig. 21.17. Thus, the thermal conductivity

Fig. 21.15 Composite plates for thermal insulation test (**a**) without a render and (**b**) with two layers of render

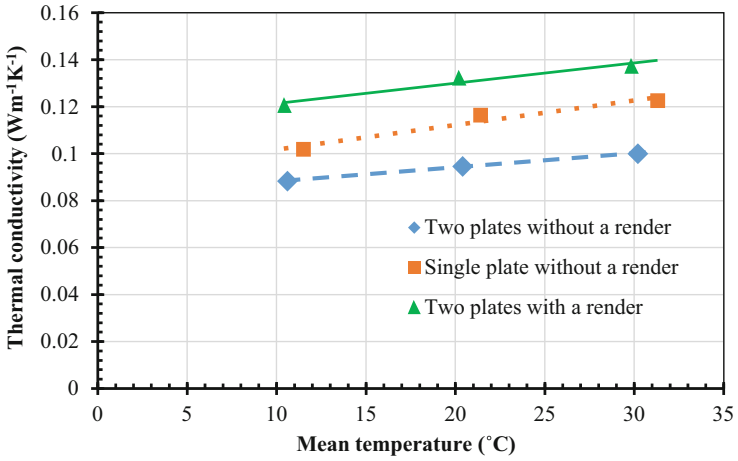


Fig. 21.16 Thermal conductivity of the biocomposite

value obtained for the biocomposite is suitable for its application in building thermal insulation. Moreover, it has advantages over conventional insulation materials due to its lower production costs and environmental impacts. Moreover, it has the crucial advantage being able to grow into the desired form when guided by a formwork.

21.3.6 Combustion Behaviour

Small-scale fire tests were carried out for the evaluation of the combustion behaviour of the new mycelium-based biocomposite according to the standard EN 13501-2: 2003 [80]. The fire test was started with a plate without render. It took 40 min before it caught fire. Figure 21.18 shows the temperature recorded at two points on the plate during the fire test. The dashed line corresponds to the temperatures measured at the

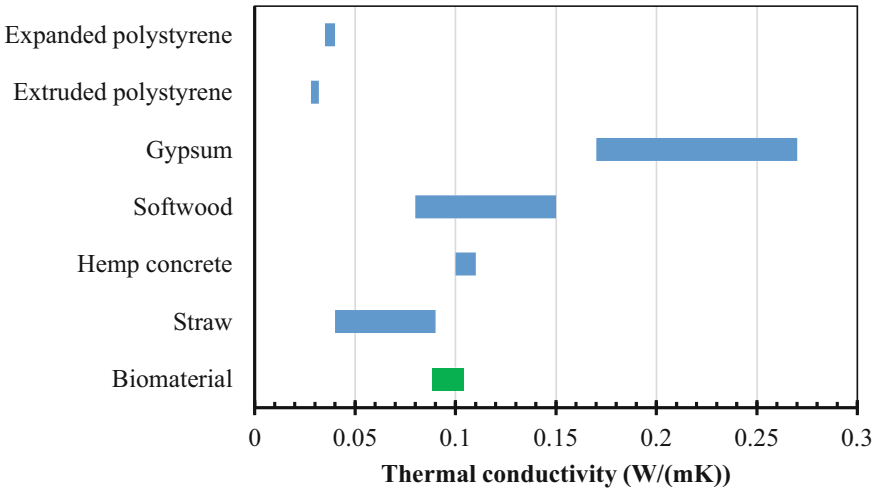


Fig. 21.17 Comparison of the thermal conductivity of the biocomposite with conventional insulation materials. Sources [76–79]

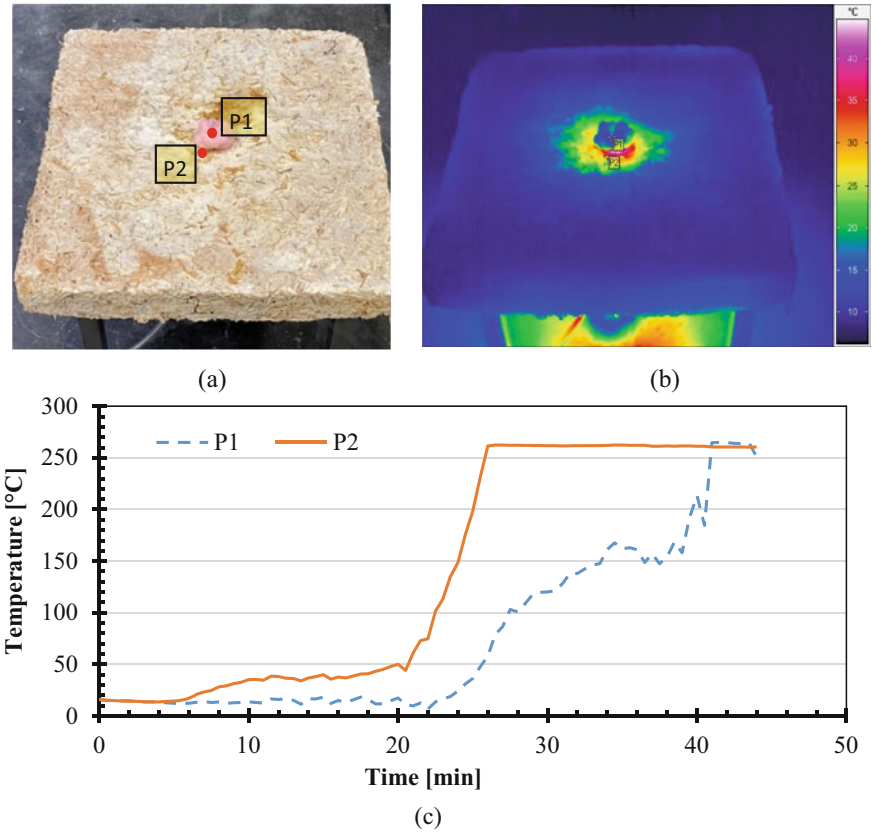


Fig. 21.18 (**a**) Measurement points P1 and P2, (**b**) captured temperature at P1 and P2 and (**c**) variation of temperature at P1 and P2 [74]

Fig. 21.19 Bottom faces of (**a**) plate without a render and (**b**) plate with a render, after the fire test

cotton wool (P1), while the solid line corresponds to the temperature measured on top of the plate just close to the cotton wool (P2). It was observed that the temperature at point P1 slightly decreased till 22 min. This is due to the high smoke created during the test that affected the measurement procedure.

Another test was conducted on a plate with render. Since it showed no combustion, the test was subsequently stopped after 70 min. However, it was observed that the bottom of the plate was burnt. Figure 21.19 shows the bottom faces of the samples after each test. Based on these test results, the fire resistance of category EI15 according to the standard EN13501-2:2003 [80] was measured for the new biocomposite.

21.4 Conclusions

Research on mycelium-based biocomposites is becoming popular in recent times due to their renewable and biodegradable nature as well as low density and hygrothermal properties. The use of agricultural crop by-products is one of the key strategies to reduce the use of non-renewable resources. Mycelium-based biocomposites, which require lesser energy and lower costs, are sustainable and renewable and they can also replace traditional polymer-based insulation materials.

Research on mycelium-based biocomposites is ongoing, and some technical gaps still need to be filled to develop these composites to obtain the desired hygrothermal properties. This study focused on the development of a new biocomposite using two fungi, *Ganoderma resinaceum* and *Pleurotus ostreatus*, grown on *Miscanthus* fibres as an economical and sustainable alternative material for traditional building insulation materials. The data suggested a promising new biocomposite for use in indoor building thermal applications in comparison with conventional insulation materials.

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References

1. Mohanty AK, Misra M, Hinrichsen G (2000) Biofibres, biodegradable polymers and biocomposites: an overview. Macromol Mater Eng 276:1–24
2. Palumbo M, Avellaneda J, Lacasta AM (2015) Availability of crop by-products in Spain: new raw materials for natural thermal insulation. Resour Conserv Recycl 99:1–6
3. Korjenic A, Petranek V, Zach J, Hroudova J (2011) Development and performance evaluation of natural thermal-insulation materials composed of renewable resources. Energ Buildings 43: 2518–2523
4. Schiavoni S, D'Alessandro F, Bianchi F, Asdrubali F (2016) Insulation materials for the building sector: a review and comparative analysis. Renew Sust Energ Rev 62:988–1011
5. Xu J, Sugawara R, Widyorini R, Han G, Kawai S (2004) Manufacture and properties of low-density binderless particleboard from kenaf core. J Wood Sci 50:62–67
6. Pinto J, Cruz D, Paiva A, Pereira S, Tavares P, Fernandes L, Varum H (2012) Characterization of corn cob as a possible raw building material. Constr Build Mater 34:28–33
7. Gaujena B, Agapovs V, Borodinecs A, Strelets K (2020) Analysis of thermal parameters of hemp fiber insulation. Energies 13:6385
8. Vo LTT, Navard P (2016) Treatments of plant biomass for cementitious building materials – a review. Constr Build Mater 121:161–176
9. Schnabel T, Huber H, Petutschnigg A, Jäger A (2019) Analysis of plant materials pre- treated by steam explosion technology for their usability as insulating materials. Agron Res 17(S1): 1191–1198
10. Eschenhagen A, Raj M, Rodrigo N, Zamora A, Labonne L, Evon P, Welemane H (2019) Investigation of miscanthus and sunflower stalk fiber-reinforced composites for insulation applications. Adv Civ Eng 9328087. https://doi.org/10.1155/2019/9328087
11. El Hage R, Khalaf Y, Lacoste C, Nakhl M, Lacroix P, Bergeret A (2018) Aflame retarded chitosan binder for insulating miscanthus/recycled textile fibers reinforced biocomposites. J Appl Polym Sci 136:47306
12. Abhijith R, Ashok A, Rejeesh CR (2018) Sustainable packaging applications from mycelium to substitute polystyrene: a review. Mater Today: Proc 5:2139
13. Jiang L, Walczyk D, McIntyre G, Chan WK (2016) Cost modeling and optimization of a manufacturing system for mycelium-based biocomposite parts. J Manuf Syst 41:8–20
14. Jones M, Huynh T, Dekiwadia C, Daver F, John S (2017) Mycelium composite: a review of engineering characteristics and growth kinetics. J Bionanosci 11(4):241–257
15. Arifin YH, Yusuf Y (2013) Mycelium fibres as new resource for environmental sustainability. Proc Eng 53:504–508
16. Holt G, Mcintyre G, Flagg D, Bayer E, Wanjura J, Pelletier M (2012) Fungal mycelium and cotton plant materials in the manufacture of biodegradable molded packaging material: evaluation study of select blends of cotton byproducts. J Biobased Mater Bioenergy 6(4):431–439
17. Haneef M, Ceseracciu L, Canale C, Bayer IS, Heredia-Guerrero JA, Athanassiou A (2017) Advanced materials from fungal mycelium: fabrication and tuning of physical properties. Sci Rep 41292:7
18. Hawksworth DL, Lücking R (2017) Fungal diversity revisited: 2.2 to 3.8 million species. Microbiol Spectr 5(4). https://doi.org/10.1128/microbiolspec.FUNK-0052-2016
19. Smith M, Bruhn J, Anderson J (1992) The fungus armillaria bulbosa is among the largest and oldest living organisms. Nature 356:428–431
20. Webster J, Weber R (2007) Introduction to fungi. Cambridge University Press, Cambridge

21. Wösten HAB (2019) Filamentous fungi for the production of enzymes, chemicals and materials. Curr Opin Biotechnol 59:65–70
22. Bonfante P, Genre A (2010) Mechanisms underlying beneficial plant-fungus interactions in mycorrhizal symbiosis. Nat Commun 1:48
23. Islam MR, Tudryn G, Bucinell R, Schadler L, Picu RC (2017) Morphology and mechanics of fungal mycelium. Sci Rep 7:13070
24. Jiang L, Walczyk D, McIntyre G (2017) A new approach to manufacturing biocomposite sandwich structures: investigation of preform shell behavior. J Manuf Sci Eng 139(2):021014
25. Grimm D, Wösten HAB (2018) Mushroom cultivation in the circular economy. Appl Microbiol Biotechnol 102:7795–7803
26. Chang AC (1992) Genetics and breeding of edible mushrooms. CRC Press, Boca Raton
27. Lelivelt RJJ, Lindner G, Teuffel P, Lamers H (2015) The production process and compressive strength of mycelium-based materials. First international conference on bio-based building materials, Clermont-Ferrand
28. Walker GM, White NA (2018) Introduction to fungal physiology. In: Fungi: biology and applications, 3rd edn. Wiley, Hoboken, pp 1–34
29. Jones M, Bhat T, Wang CH, Moinuddin K, John S (2017) Thermal degradation and fire reaction properties of mycelium composites. 21st international conference on composite materials, Xi'an
30. Elsacker E, Vandelook S, Brancart J, Peeters E, De Laet L (2019) Mechanical, physical and chemical characterisation of mycelium-based composites with different types of lignocellulosic substrates. PLoS One 14(7):e0213954
31. Ghazvinian A, Farrokhsiar P, Vieira F, Pecchia J, Gursoy B (2019) Mycelium-based bio-composites for architecture: assessing the effects of cultivation factors on compressive strength. eCAADe and SIGraDi conference, Porto
32. Zimele Z, Irbe I, Grinins J, Bikovens O, Verovkins A, Bajare D (2020) Novel mycelium-based biocomposites (MBB) as building materials. J Renew Mater 8(9):1067–1076
33. Pelletier MG, Holt GA, Wanjura JD, Bayer E, McIntyre G (2013) An evaluation study of mycelium based acoustic absorbers grown on agricultural by-product substrates. Ind Crop Prod 51:480–485
34. Bruscato C, Malvessi E, Brandalise RN, Camassola M (2019) High performance of macrofungi in the production of mycelium-based biofoams using sawdust - sustainable technology for waste reduction. J Clean Prod 234:225–232
35. Modanloo B, Ghazvinian A, Matini M, Andaroodi E (2021) Tilted arch; implementation of additive manufacturing and bio-welding of mycelium-based composites. Biomimetics 6(4):68
36. Tudryn GJ, Smith LC, Freitag J, Bucinell R, Schadler LS (2018) Processing and morphology impacts on mechanical properties of fungal based biopolymer composites. J Environ Polym Degrad 26(4):1–11
37. Meng L, Fu Y, Li D, Sun X, Chen Y, Li X, Xu S, Li X, Li C, Song B, Li Y (2019) Effects of corn stalk cultivation substrate on the growth of the slippery mushroom (Pholiota microspora). RSC Adv 9:5347–5353
38. Chen B, Ke B, Ye L, Jin S, Jie F, Zhao L, Wu X (2017) Isolation and varietal characterization of Ganoderma resinaceum from areas of Ganoderma lucidum production in China. Sci Hortic 224: 109–114
39. Hoa HT, Wang C-L (2015) The effects of temperature and nutritional conditions on mycelium growth of two oyster mushrooms (Pleurotus ostreatus and Pleurotus cystidiosus). Mycobiology 43(1):14–23
40. Ziegler AR, Bajwa SG, Holt GA, McIntyre G, Bajwa DS (2016) Evaluation of physico-mechanical properties of mycelium reinforced green biocomposites made from cellulosic fibers. Appl Eng Agric 32(6):931–938
41. Yang Z, Zhang F, Still B, White M, Amstislavski P (2017) Physical and mechanical properties of fungal mycelium-mased biofoam. J Mater Civ Eng 29(7):04017030
42. Van Horn WM, Shema BF, Shockley WH, Conkey JH (1957) Sheets comprising filament of fungi. US Patent US2811442A

43. Johnson MA, Carlson JA (1978) Mycelial paper: a potential resource recovery process? Biotechnol Bioeng 20(7):1063–1084
44. Silverman J, Cao H, Cobb K (2020) Development of mushroom mycelium composites for footwear products. Cloth Text Res J 38(2):119–133
45. Jones M, Gandia A, John S, Bismarck A (2020) Leather-like material biofabrication using fungi. Nat Sustain 4:9–16
46. Travaglini S, Noble J, Ross PG, Dharan CKH (2013) Mycology matrix composites. 28th technical conference. American Society for Composites
47. He J, Cheng C-M, Su D-G, Zhong M-F (2014) Study on the mechanical properties of the latex-mycelium composite. Appl Mech Mater 507:415–420
48. Mayoral Gonzalez E, Gonzalez Diez I (2015) Bacterial induced cementation processes and mycelium panel growth from agricultural waste. Key Eng Mater 663:42–49
49. López Nava JA, Méndez González J, Ruelas Chacón X, Nájera Luna JA (2016) Assessment of edible fungi and films bio-based material simulating expanded polystyrene. Mater Manuf Process 31(8):1085–1090
50. Pelletier MG, Holt GA, Wanjura JD, Lara AJ, Tapia-Carillo A, Mclntyre G, Bayer E (2017) An evaluation study of pressure-compressed acoustic absorbers grown on agricultural by-products. Ind Crop Prod 95:342–347
51. Dahmen J (2017) Soft futures: mushrooms and regenerative design. J Archit Educ 71(1):57–64
52. Attias N, Danai O, Ezov N, Tarazi E & Grobman YJ (2017) Developing novel applications of mycelium based biocomposite materials for design and architecture. Building with bio-based materials: best practice and performance specification, Croatia
53. Campbell S, Correa D, Wood D & Menges A (2017) Modular mycelia: scaling fungal growth for architectural assembly. eCAADe regional international symposium, Wales
54. Jones M, Bhat T, Huynh T, Kandare E, Yuen R, Wang CH, John S (2018) Waste- derived low-cost mycelium composite construction materials with improved fire safety. Fire Mater 42(7):816–825
55. Appels FVW, Camere S, Montalti M, Karana E, Jansen KMB, Dijksterhuis J, Krijgsheld P, Wosten HAB (2019) Fabrication factors influencing mechanical, moisture- and water-related properties of mycelium-based composites. Mater Des 161:64–71
56. Sun W, Tajvidi M, Hunt CG, McIntyre G, Gardner DJ (2019) Fully bio-based hybrid composites made of wood, fungal mycelium and cellulose nanofibrils. Sci Rep 9:3766
57. Matos MP, Teixeira JL, Nascimento BL, Griza S, Holanda FSR, Marino RH (2019) Production of biocomposites from the reuse of coconut powder colonized by Shiitake mushroom. Sci Agrotechnol 43:e003819
58. Attias N, Danai O, Tarazi E, Pereman I, Grobman YJ (2019) Implementing bio-design tools to develop mycelium-based products. Des J 22(1):1647–1657
59. Ridzqo IF, Susanto D, Panjaitan TH, Putra N (2020) Sustainable material: development experiment of bamboo composite through biologically binding mechanism. IOP Conf Ser Mater Sci Eng 713(1):012010
60. Soh E, Chew ZY, Saeidi N, Javadian A, Hebel D, Le Ferrand H (2020) Development of an extrudable paste to build mycelium-bound composites. Mater Des 195:109058
61. Ding R, Yang X, Peng L, Xie J, Chen C (2021) Development of mycelium materials incubating Pleurotus ostreatus fungi with different substrates composed of poplar sawdust and cottonseed hull. Research Square. https://doi.org/10.21203/rs.3.rs-960342/v1
62. Cerimi K, Akkaya KC, Pohl C, Schmidt B, Neubauer P (2019) Fungi as source for new bio-based materials: a patent review. Fungal Biol Biotechnol 6:17
63. Cadena CG, Silvera AJB (2002) Estudio de la variación en la conductividad térmica de la cascarilla de arroz aglomerada con fibras vegetales. Ingeniería y Desarrollo 12:8–9
64. Dominguez-Munoz F, Anderson B, Cejudo-Lopez JM, Carrillo-Andres A (2010) Uncertainty in the thermal conductivity of insulation materials. Energ Buildings 42(11):2159–2168
65. Norford LK, Glicksman LR, Harvey HS, Charlson JA (1999) Development of low- cost wheat-straw insulation board. HVAC & R Res 5(3):249–263

66. Xing Y, Brewer M, EI-Gharabawy H, Griffith G, Jones P (2018) Growing and testing mycelium bricks as building insulation materials. IOP Conf Ser Earth Environ Sci 121:022032
67. Wimmers G, Klick J, Tackaberry L, Zwiesigk C, Egger K, Massicotte H (2019) Fundamental studies for designing insulation panels from wood shavings and filamentous fungi. Bioresources 14:5506–5520
68. Schritt H, Vidi S, Pleissner D (2021) Spent mushroom substrate and sawdust to produce mycelium-based thermal insulation composites. J Clean Prod 313(1):127910
69. Pereira Dias P, Waldmann D (2021) Optimisation of the mechanical properties of Miscanthus lightweight concrete. Constr Build Mater 258:119643
70. Pude R, Treseler CH, Noga G (2004) Morphological, chemical and technical parameters of miscanthus genotypes. J Appl Bot Food Qual 78:58–63
71. Courard I, Darimont A, Louis A, Michel F (2011) Mineralization of bio-based materials: effect on cement-based mix properties. Bulletin of the Polytechnic Institute of Jassy, Construction. Architecture Section
72. Pereira Dias P, Kammer VJ, Waldmann D (2021) Analysis of the geometrical imperfections of a dry-stacked masonry block based on Miscanthus. Constr Build Mater 310:125282
73. Pereira Dias P, Jayasinghe LB, Waldmann D (2021) Machine learning in mix design of Miscanthus lightweight concrete. Constr Build Mater 302:124191
74. Pereira Dias P, Jayasinghe LB, Waldmann D (2021) Investigation of mycelium-Miscanthus composites as building insulation material. Results Mater 10:100189
75. EOTA (2013) ETAG 004. European Organisation for Technical Approvals, Brussels
76. Pruteanu M (2010) Investigations regarding the thermal conductivity of straw. The Bulletin of the Polytechnic Institute of Jassy, Construction. Architecture Section, vol 3, pp 9–16
77. Collet F, Pretot S (2014) Thermal conductivity of hemp concretes: variation with formulation, density and water content. Constr Build Mater 65(1):612–619
78. Bergman TL, Incropera FP, De Witt DP, Lavine AS (2011) Fundamentals of heat and mass transfer. Wiley, Hoboken
79. Margit P, Roland G, Martins HS & Alexander R (2008) Insulating materials: principle, materials, applications. Birkhauser Verlag
80. EN 13501-2 (2003) Fire classification of construction products and building elements - part 2: classification using data from fire resistance tests, excluding ventilation services. European Committee for Standardization, Brussels

Index

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